26791

National Library of Canada | Bibliothèque nationale du Canada

CANADIAN THESES ON MICROFICHE | THÈSES CANADIENNES SUR MICROFICHE

NAME OF AUTHOR/*NOM DE L'AUTEUR* Allen M. Jobson.

TITLE OF THESIS/*TITRE DE LA THÈSE* Physiological Characterization of a Desulfovibrio species Isolated from Crude Oil

UNIVERSITY/*UNIVERSITÉ* Alberta

DEGREE FOR WHICH THESIS WAS PRESENTED/*GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE* PhD.

YEAR THIS DEGREE CONFERRED/*ANNÉE D'OBTENTION DE CE GRADE* 1975

NAME OF SUPERVISOR/*NOM DU DIRECTEUR DE THÈSE* Dr. D.W.S. Westlake

Permission is hereby granted to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

L'autorisation est, par la présente, accordée à la BIBLIOTHÈQUE NATIONALE DU CANADA de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans l'autorisation écrite de l'auteur.

DATED/*DATÉ* Oct 22 SIGNED/*SIGNÉ* Allen M. Jobson.

PERMANENT ADDRESS/*RÉSIDENCE FIXE*
Research Council of Alberta
87 Ave & 113 St.
Edmonton, Alberta.

NL-91 (3-74)

THE UNIVERSITY OF ALBERTA

Physiological Characterization of a Desulfovibrio sp. Isolated from Crude Oil

BY

© Allen M. Jobson (BSc. Ag., MSc. Micro.)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

FALL, 1975

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled

"Physiological characterization of a Desulfovibrio sp.
Isolated from Crude Oil"

submitted by Allen M. Jobson in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

D W S Westlake ....

Fred D Cook ....
Supervisors

J N Campbell ....

W. H. Tyler ....

Rita R. Colwell ....
External Examiner

Date ....Oct. 3........ 1975

- This thesis is dedicated to my

Father, Lester C. Jobson (1910 - 1975) -

ABSTRACT

A sulfate reducing bacterial isolate capable of growing on modified Butlin's medium was enriched from a sample of North Cantal oil. The purified isolate was found to be a non-spore forming, vibrio-shaped rod. Based on its G+C content and physiological characteristics the isolate was provisionally classified as Desulfovibrio vulgaris var. oxamicus.

Initial chemostat studies were concerned with the effect of media composition at constant growth rate, on the macromolecular composition of the cells. The utilization of lactate and sulfate, the production of reduced sulfur intermediates and sulfide, and isotopic fractionation of sulfur were also investigated. A medium was formulated based on these results which was used in studying the effect of growth rate on the above parameters. The results indicate that the macromolecular composition of the cells is dependent on the growth conditions and is not under as tight a metabolic control as in organisms such as Escherichia coli or Salmonella typhimurium. The utilization of lactate and sulphate and the production of reduced sulfur intermediates and sulphide were also affected by growth rate.

The incorporation of ^{14}C-labelled yeast protein hydrolysate into cell components was investigated under different cultural conditions. As very little radioactivity was incorporated into cellular material or released as CO_2 it is probable that yeast extract functions as a stimulant of lactate metabolism but is not itself

actively used for cell synthesis.

The role of sulphate reducing microorganisms in the metabolism of oil was investigated under field and laboratory conditions. Laboratory studies were carried out to determine the ability of pure and mixed cultures of sulphate reducing microorganisms to grow and metabolize North Cantal crude oil under anaerobic conditions. Growth was determined by blackening of the medium. The oil was recovered and analysed chemically for changes in composition and the saturate components examined by a GLC technique for alterations in the n-alkane profile. None of the parameters investigated resulted in any utilization of the oil by the sulphate reducing cultures tested. The inability to use n-alkanes was confirmed in experiments using media containing ^{14}C-labelled octadecane. There was no assimilation of label, no production of $^{14}CO_2$ or of radioactive intermediates such as octadecanoic acid. Both the sulfate reducing isolate and enriched mixed cultures containing sulfate-reducers were able to respire on spent aerobic cultures which had originally used crude petroleum as their sole source of carbon and energy.

The examination of the numbers of sulfate reducing microorganisms in soil samples from oil spill test plots were investigated. Increased numbers of sulfate-reducers were found in many of the plots which had received chemical fertilizer amendments. It was not possible, however, to directly implicate these organisms in the degradation of oil which was known to have taken place in many of the plots examined.

The results of these studies are compatible with the hypothesis that these sulfate-reducers function as a succession population, that is, they are not able to grow on oil 'per se' but are able to grow on the products of the aerobic catabolism of oil. This hypothesis would explain their increased incidence in and around natural oil deposits.

ACKNOWLEDGEMENTS

The author wishes to express sincere gratitude to Dr. D.W.S. Westlake for his direction during the course of these studies and Dr. F.D. Cook for his keen observations concerning sulfate reducing microorganisms. Their backgrounds of intermediary metabolism, continuous culture, and soil ecology have lent a great deal to the scope of this project.

The author acknowledges the financial support of the National Research Council of Canada, and the University of Alberta, without which this work would not have been possible.

Numerous people in the Department of Microbiology have assisted at various points during the course of the research; their help and encouragement is very gratefully acknowledged.

The able assistance of Dr. H.R. Krouse and his staff at the University of Calgary, in the mass spectrometric analysis of sulfur samples, is acknowledged and was sincerely appreciated.

Finally the author expresses sincere gratitude to his wife Lynn for her concern, understanding, and moral support throughout this time in graduate studies.

TABLE OF CONTENTS

Page

ABSTRACT iv

ACKNOWLEDGEMENTS vii

LIST OF TABLES xii

LIST OF FIGURES xiv

LIST OF PLATES xvi

LIST OF ABBREVIATIONS xvii

INTRODUCTION 1

LITERATURE REVIEW 3

I. Introductory Remarks 3

II. Sulfur Isotope Fractionation by Dissimilatory Sulfate Reducing Bacteria 5

III. Carbon and Electron-Donating Substrates 7

IV. Carbon Dioxide Fixation and Mixotrophy in *Desulfovibrio* sp. 11

V. Other Carbon Sources Utilized by *D. desulfuricans* (strain Hildenborough) 12

VI. The Utilization of Hydrocarbons by Dissimilatory Sulfate-Reducers and Their Role in Crude Petroleum Degradation 13

VII. Syntrophy Between Sulfate-Reducers and Other Bacteria 20

VIII. Mechanism of Dissimilatory Sulfate Reduction 20

IX. Hydrogen Metabolism in *Desulfovibrio* sp. 28

X. Interspecies Hydrogen Transfer 30

XI. Growth Yield Studies of *Desulfovibrio* sp. 31

Page

MATERIALS AND METHODS 34

I. Sources of Chemicals, Reagents, Solvents, and Petroleum 34

II. Microbiological 34
- A. Cultures Employed 34
- B. Media 34
- C. Culture Maintenance 37
- D. Enumeration of Sulfate-Reducers 37
- E. Electron Microscopy 38

III. Culturing Techniques 38
- A. Batch Culture 38
- B. Continuous Culture 39

IV. Analytical Methods 40
- A. Cellular Analyses 40
  - (i) Cell Fractionation 40
  - (ii) Protein 41
  - (iii) RNA 41
  - (iv) DNA 42
  - (v) DNA Isolation 42
  - (vi) Percent Guanosine and Cytosine 42
  - (vii) Lipid Extraction and Methylation 43
  - (viii) Cytochrome Difference Spectrum 44
- B. Chemical Analyses 44
  - (i) Sulfate 44
  - (ii) Lactate 44
  - (iii) Iodimetric Determination of Sulfite, Sulfide, and Thiosulfate 45
- C. Isotope Fractionation 46
  - (i) Chemical Reduction of Samples 46
  - (ii) Sample Burning 47
  - (iii) Mass Spectrometry 47

V. ^{14}C Radioactive Techniques 48
- A. Reaction Vessels 48
- B. Labelled Substrates 49
  - (i) Lactate 49
  - (ii) Yeast Extract 49
  - (iii) Stearic Acid 49
  - (iv) Octadecane 49
- C. Scintillation Counting 49

VI. Chromatographic Techniques 50
- A. Thin Layer Chromatography 50
  - (i) Lactate 50
  - (ii) Stearic Acid 50

Page

(iii) Octadecane 50

B. Liquid Chromatography of Crude Petroleum 51

C. Gas Chromatography 51
(i) Fatty Acid Methyl Esters 51
(ii) Petroleum Saturates 52

RESULTS AND DISCUSSION 53

I. Physiological Characteristics of an Unidentified, Purified, Sulfate Reducing Isolate 53
A. Enrichment of the Organism from North Cantal Oil . 53
B. Determination of the Purity of Isolate #1 57
C. Buoyant Density Measurements of DNA Extracted from Isolate #1 57
D. The Relationship of the Percent Guanosine + Cytosine Content of Isolate #1 to known *Desulfovibrio* sp. 62
E. Effect of Temperature on the Respiration of Isolate #1 67
F. Determination of the Cytochrome Spectrum of Isolate #1 67
G. Studies Concerning the Response of Isolate #1 to Alternate Electron Donors 72
H. Attempts to Extract Alkanes from Isolate #1 75
I. Fatty Acid Composition of the Lipid Component of Isolate #1 75

II. Effects of Levels of Electron Donor and Electron Acceptor on the Growth and Metabolism of Isolate #1 Grown in Continuous Culture at a Constant Dilution Rate 81
A. Consumption of Lactate and Sulfate by Isolate #1 as a Function of Influent Medium 83
B. Production of Cellular Constituents 85
C. Growth Yield Calculations 90
D. Production of Reduced Sulfur Intermediates as a Function of the Composition of the Influent Growth Medium 94
E. Detection of ^{34}S Enrichment in Residual Sulfate as a Function of Influent Growth Medium Composition 102

III. Effect of Dilution Rate on the Growth of Isolate #1 under Continuous Culturing Conditions 105
A. Growth Conditions 105
B. Growth of Isolate #1 as Measured by Viable Counts and the Production of Protein, RNA, and DNA as a Function of Chemostat Dilution Rate 110

Page

(i) Viable Counts 110
(ii) Protein Production 110
(iii) RNA Production 117
(iv) DNA Production 120

C. Cell Size Distribution of Isolate #1 as a Function of Dilution Rate 126

D. The Production of Intermediary Metabolites and End-Products of Dissimilatory Sulfate Reduction as a Function of Dilution Rate 126
(i) Solution and Sparged Sulfide Levels 130
(ii) Sulfite Production 135
(iii) Thiosulfate Production 138
E. Sulfur Recoveries 141

F. Sulfur Isotope Fractionation 143

IV. Incorporation of Uniformly ^{14}C-Labelled Yeast Protein Hydrolysate Into Batch Cultures of Isolate #1 146

V. Investigations Concerning the Role of Dissimilatory Sulfate Reducers in Crude Oil Catabolism 148
A. Incidence of Sulfate Reducing Microorganisms in Areas of Terrestrial Oil Spills 150
B. *In vitro* Studies on Petroleum Degradation by Isolate #1 and by Mixed Cultures #1 and #2 154
(i) Test Tube Cultures of Isolate #1 in the Presence of Crude Oil 154
(ii) Growth of Isolate #1 and Mixed Cultures MC #1 and MC #2 in the Presence of North Cantal Oil in Sealed, Agitated Flasks 159
C. Metabolism of Mono-Terminally-Labelled ^{14}C-Octadecane by Isolate #1 161
D. Demonstration of Metabolism or Growth of *Desulfovibrio* sp. in Association with Aerobic Microbial Catabolism of Crude Petroleum 166
(i) Metabolism of Isolate #1 in Aerobic Mixed Cultures Growing on North Cantal Oil 166
(ii) Enrichment of a Mixed Culture of Dissimilatory Sulfate Reducing Microorganisms from Norman Wells Refinery Soil 171
(iii) Evaluation of the Medium used by ZoBell to Demonstrate Crude Oil Catabolism 175

CONCLUSIONS 179

BIBLIOGRAPHY 182

APPENDIX 190

LIST OF TABLES

Table		Page
I.	Percent G + C Content Values of Members of Genus Desulfovibrio as Calculated from Buoyant Density Measurements using Different Formulae	65
II.	Summary of Amounts of H_2S Produced as a Function of Alternate Electron Donor Used After 5- and 10-Day Incubation Periods	73
III.	Cell Protein Concentrations During Growth on Medium #3 and #4	77
IV.	Concentrations of Five Identified Fatty Acids Extracted from Cultures of Isolate #1 Grown on Medium #3 as a Function of Culture Age	78
V.	Unidentified Lipid Components Extracted From Isolate #1 Grown on Medium #3 as a Function of Culture Age	79
VI.	Concentrations of Five Identified Fatty Acids Extracted from Cultures of Isolate #1 Grown on Medium #4 as a Function of Culture Age	81
VII.	Unidentified Lipid Components Extracted from Isolate #1 Grown on Medium #4 as a Function of Culture Age	82
VIII.	Consumption of Lactate and Sulfate as a Function of Influent Medium Composition	84
IX.	Production of Cellular Constituents as a Function of the Composition of the Influent Medium	86
X.	Protein: Nucleic Acid Ratios as a Function of the Composition of the Influent Medium	88
XI.	Viable Count as a Function of the Composition of the Influent Growth Medium	89
XII.	Summary of Cellular Length (µm) and Half-Cycle Distribution as a Function of the Composition of the Influent Growth Medium	91
XIII.	Molar Protein Yields as a Function of the Composition of the Influent Medium	95

Table Page

XIV. Production Levels of Effluent Sulfide, Sulfite, Thiosulfate and Sparged H_2S as a Function of the Composition of the Influent Medium 96

XV. Sulfur Recoveries during Growth on the Five Media 100

XVI. $\delta^{34}S$ Values of Influent and Effluent Sulfate-Sulfur as a Function of the Growth Medium Composition 103

XVII. Summary of Cell Size Distribution and Half-Cycle Frequency as a Function of Chemostat Dilution Rate . . . 127

XVIII. Inflow and Outflow Recoveries of Sulfur as a Function of Dilution Rate 142

XIX. $\delta^{34}S$ Values for Influent and Effluent Sulfate as a Function of Dilution Rate 144

XX. Incorporation of ^{14}C-labelled Yeast Protein Hydrolysate Material into Cellular Components of Isolate #1 (2×10^6 dpm/culture) 147

XXI. Percent of the ^{14}C-Labelled Protein Hydrolysate Incorporated into Cellular Components or Found in Metabolic CO_2 149

XXII. Summary of Sulfate-Reducer Viable Counts (M.P.N.) in Samples Taken from Oil Spill Plots in Northern Alberta 665 Days After Spill Initiation 151

XXIII. T-Test of Data from Table XXII, Comparing Test Plot Counts to Control Plot Counts 153

XXIV. Results of the Tube Culture of Isolate #1 in the Presence of North Cantal Crude Petroleum 155

XXV. Details of Culturing Experiment Using Sealed, Agitated Flasks 160

XXVI. Summary of Hydrogen Sulfide Production and Liquid Chromatographic Analyses of Residual Oil Extracted from the Sealed Shake Flask Experiment 164

XXVII. Incorporation of ^{14}C-1-Labelled Lactate Into Isolate #1 Cellular Components and Co-Production Levels of $^{14}CO_2$ (1.0×10^6 dpm/culture) 191

XXVIII. Percent of the ^{14}C-1 Labelled Lactate Incorporated Into Cellular Components or Found in Metabolic CO_2 192

LIST OF FIGURES

Figure Page

1. Densitometer Tracings and Buoyant Density Values of DNA from Isolate #1 as determined by Equilibrium Centrifugation 64

2. Hydrogen Sulfide Production of Isolate #1 as a Function of Incubation Temperature 69

3. Reduced Minus Oxidized Difference Spectrum of a Whole Cell Preparation of Isolate #1 71

4. Size Distribution of Isolate #1 Cells as a Function of Growth Medium 93

5. Forms of Soluble Sulfides in Aqueous Solution as a Function of pH 99

6. Flow Rate Characteristics of the Chemapac Peristaltic Pump used in the Dilution Rate Study . . . 107

7. pH of the Chemostat Effluent Flow as a Function of Dilution Rate 109

8. Viable Count per Milliliter of Culture and Total Viable Count per Hour as a Function of Dilution Rate . 112

9. Cellular Protein per Milliliter, per Hour, and per Viable Cell as a Function of Dilution Rate 114

10. RNA-Ribose per Milliliter and per Hour and RNA per Viable Cell as a Function of Dilution Rate 119

11. Deoxy-d-Ribose per Milliliter and per Hour and DNA per Viable Cell as a Function of Dilution Rate 122

12. Protein: RNA, Protein: DNA and RNA: DNA Ratios as a Function of Dilution Rate 125

13. Size Distribution of Isolate #1 Cells as a Function of Dilution Rate 129

14. Micromoles per Milliliter of Effluent and Total Production per Hour of Solution Sulfide as a Function of Dilution Rate 132

15. Micromoles of Hydrogen Sulfide Sparged from the Chemostat as a Function of Dilution Rate 134

Figure Page

16. Namomoles of Sulfite per Milliliter of Chemostat Effluent and Total Micromoles of Sulfite produced per Hour as a Function of Dilution Rate 137

17. Nanomoles of Thiosulfate per Milliliter of Chemostat Effluent and Total Micromoles of Thiosulfate Produced per Hour as a Function of Dilution Rate 140

18. Gas Chromatographic Profiles of the Saturate Fraction from Residual North Cantal Crude Oil Extracted from the Tube Incubation Experiment 158

19. GLC Saturate Profiles of the Residual Oil Samples Extracted from the Sealed Shake Flasks After 112 Days Incubation 163

LIST OF PLATES

Plate		Page
I.	Experimental Design used to Enrich Sulfate-Reducing Organisms from North Cantal Petroleum	56
II(a)	Electron Micrographs of Negatively-Stained Preparations of Isolate #1	59
II(b)	Electron Micrographs of Negatively-Stained Preparations of Isolate #1	61
III.	Metabolism by Isolate #1 on Spent, Aerobic Oil-Utilizing Culture	169
IV.	Photograph of Bottles After Twenty Days Incubation at 30° Demonstrating H_2S Production	173
V.	Photograph of Growth Flasks After 72 Hours Incubation at 30° Using ZoBell's Growth Medium	178

ABBREVIATIONS

APS	adenosine 5'-sulfatophosphate
EGS	ethylene glycol succinate
E'_o	redox potential at pH 7.0
hr^{-1}	per hour (form restricted to the description of dilution rate in chemostate studies)
OV-101	methyl silicone
o/oo	per mil
PAPS	Adenosine 3'-phosphate 5'-sulfatophosphate

INTRODUCTION

It is a well-established fact that sulfate-reducing microorganisms exert a profound influence on the overall environment, especially with respect to the sulfur cycle. The role of this class of microorganisms in the genesis of hydrocarbon deposits has been clearly established by previous workers. However, the utilization of hydrocarbons by sulfate-reducing microorganisms has not been a straightforward, clearly- and consistently-demonstrable fact as is the case with aerobic utilization of hydrocarbons. Previous studies by Jobson et al (1972), Bailey et al (1973), demonstrated that in vitro degradation of crude petroleum by mixed aerobic populations gave patterns of degradation similar to those seen in an actual oil deposit in the Province of Saskatchewan. Degradation of n-paraffins and some aromatic components coupled with increases in the specific gravity of the crude petroleum were the main changes observed. The data at that time fitted an aerobic model since it was known that fresh groundwater wash was occurring in the region where severe degradation had occurred. However, there were regions within the deposit where the oil still appeared relatively unattacked; yet rock materials taken from these areas showed evidence of altered $^{34}S/^{32}S$ ratios which could not be explained using a geothermal alteration model. This made it very tempting to speculate that sulfate-reducers had either been active in the past or were presently growing and metabolizing within this region.

In the literature surveyed to date, there have been no reported studies, using liquid as gas chromatographic techniques, to directly show whether or not sulfate-reducers catabolize crude petroleum. Moreover, with the exception of one published study, there is little information dealing with the role of sulfate reducers in environments where active petroleum catabolism via classical aerobic routes is occurring.

The general physiology of sulfate-reducers has been studied by various workers over several decades; however, specific details as to rates of growth, electron donor candidates, RNA, DNA, and protein levels, etc. have not been dealt with to any extent. As well, the behavior of *Desulfovibrio* sp. in continuous culture has not been systematically studied.

Therefore, the objectives of this project were as follows:

1. to characterize a culture of *Desulfovibrio* which had been enriched from samples of a crude petroleum;
2. to investigate the growth characteristics of this isolate in continuous culture;
3. to attempt to relate the presence of dissimilatory sulfate-reducers to areas where actual oil spills had occurred;
4. to attempt to show direct or indirect catabolism of crude oil component(s) by pure or mixed cultures of *Desulfovibrio* sp.

LITERATURE REVIEW

I. Introductory Remarks

Sulfate reducing bacteria are among the most ubiquitous of organisms, being found in practically every type of soil and natural water. Holland, a country of odoriferous canals and famous microbiologists, contributed most to our early knowledge of them. They were first formally described by Beijerinck (1895), and isolated in pure culture by van Delden (1903). Baars (1930) undertook the first physiological and biochemical investigation of sulfate reducing bacteria. The work of von Wolzogen Kuhr and van der Vlugt (1934) first implicated sulfate-reducers as active agents in the corrosion of buried iron pipes. From this point on, the literature concerning sulfate-reducers was international in scope and is adequately summarized in J.R. Postgate's three reviews (1959, 1960, 1965).

Bacteria which are capable of carrying out anaerobic respiration using sulfate as a terminal electron acceptor are classified in the genus Desulfovibrio or genus Desulfotomaculum. This use of sulfate is generally termed dissimilatory sulfate reduction (Trudinger, 1969). Certain controversies exist concerning the classification of Desulfotomaculum sp. At present, the eighth edition of Bergey's Manual accepts the taxonomic criteria as put forward by L.L. Campbell (originally proposed by Campbell and Postgate, 1965) for the genus Desulfotomaculum. They are described as being chemoorganotropic, strict anaerobes, which can be either straight or curved Gram-negative rods, motile with peritrichous flagella and possessing terminal or subterminal spores. Three species are officially recognized and vary in % G+C content from 41% to 46%. At least one species, Desulfotomaculum nigrificans, results from the re-classification of Clostridium nigrificans.

Only a few organic compounds such as lactate and pyruvate act as electron donors and carbon substrates; sulfate, sulfite, and other partially oxidized sulfur compounds act as electron acceptors. They are apparently unable to utilize carbohydrates, nor can they oxidize acetate. Their growth temperature optima varies from 30° - 55° and they have a maximum temperature tolerance of 70°; therefore, they play a role in sulfate reduction in thermophilic environments.

In the seventh edition of Bergey's Manual (1957), the genus Desulfovibrio was included in the family Spirillaceae within the order Pseudomonadales. C.E. ZoBell was responsible for the classification of Desulfovibrio sp. in this manner. Since that time, a large body of evidence has accumulated which indicates a great heterogeneity within this genus. As a result, in the eighth edition of Bergey's Manual, the genus Desulfovibrio has been re-classified by J.R. Postgate within "genera of uncertain affiliation" in part nine of the section on Gram-negative anaerobic rods. Five species are officially recognized: Desulfovibrio desulfuricans, D. vulgaris, D. salexigens, D. africanus, and D. gigas. Differentiation is based chiefly on the % G+C content, presence of polar or lophotrichous flagellation, growth on pyruvate, malate, or choline-containing media with or without sulfate, and NaCl requirements. In the review by LeGall and Postgate (1973), Postgate stated that, "the taxonomic understanding of these organisms will not advance substantially until large numbers of isolates are available for study by numerical taxonomy and until reproducible tests, particularly for carbon metabolism, can be devised." Postgate also found it curious that the percentages of G+C within Desulfovibrio sp. seem to cluster within three narrowly defined values (45%, 55%, 60%) yet give an overall spread of roughly 15%. This is as opposed to most other bacterial genera which tend to spread uniformly over a moderate range.

II. Sulfur Isotope Fractionation by Dissimililatory Sulfate Reducing Bacteria

In the last 20 years there has developed an increasing interest in the fractionation of stable sulfur isotopes by microorganisms, most notably Desulfovibrio sp. This phenomenon is very relevant in any discussion pertaining to sulfur geochemistry.

Isotopic discrimination between ^{32}S and ^{34}S has been demonstrated during the reduction of sulfate to sulfide by Desulfovibrio sp. but reported values are variable. Under laboratory conditions, bacterial fractionation has given $\Delta^{34}S$ values varying from +2 o/oo to -46 o/oo, (Jones and Starkey, 1957; Harrison and Thode, 1958; Kaplan et al, 1960; Kaplan and Rittenburg, 1964; Kemp and Thode, 1968).

(NOTE) $$^{34}S \text{ o/oo} = \frac{[^{34}S/^{32}S \text{ sample} - ^{34}S/^{32}S \text{ reference}]}{^{34}S/^{32}S \text{ reference}} \times 1000$$

$\delta^{34}S$ = sample referred to meteoric sulfur

$\Delta^{34}S$ = sample referred to initial sulfate

In all these experiments, maximal enrichment of ^{34}S in residual sulfate resulted from growing the organism in the presence of excess sulfate under suboptimal growing conditions, (e.g. low temperature).

In natural, recent sediments, $\Delta^{34}S$ values of sulfide species relative to sea water sulfate were within the range of -33 o/oo to -2 o/oo, Nakai and Jensen (1964). By way of comparison Harrison and Thode (1957) found that chemical reduction of sulfate yielded a kinetic isotope effect of $\Delta^{34}S$ of -21.6 o/oo over the temperature range 18 - 50°.

Kemp and Thode (1968) confirmed in laboratory experiments with cultures of D. desulfuricans that an inverse relationship existed between the degree of fractionation and rate of reduction per cell. As well, rates were varied by changing temperature and electron donor, where the fractionation effect appeared to increase with increasing rates of reduction per cell.

Although several models have been propsed to account for the variable isotopic effects in bacterial systems, the most elaborate is that of Rees (1973). He believes that the overall fractionation is the result of additive kinetic isotope effects associated with several stages of the sulfate reduction pathway, each of which may become rate-limiting under certain conditions.

Trudinger and Chambers (1973) approached the problem in a slightly different manner. They propose that bacteria catalyze, to various degrees, isotopic exchange between sulfate and sulfide; and they state that at equilibrium this would result in $\Delta^{34}S$ values for sulfide ranging around -74 o/oo. In this same report, they give evidence which suggests (using ^{35}S) that the pathway of sulfate reduction in D. desulfuricans is reversible.

Trudinger (private communication, 1973), showed, using continuous culture methods, that fractionation was a function of the rate of sulfate reduction, i.e. ^{34}S values around -15 o/oo at high growth rates and up to -35 o/oo at low growth rates. He also found that fractionation was not affected by changes in temperature or pH. Unfortunately he also found that fractionation under a particular set of conditions was variable, most noticeably a low reduction rate. Trudinger also believes that $\Delta^{34}S$ values around -15 o/oo are very typical during biological sulfate reduction regardless of which electron donor is used. This frequently observed value is a characteristic, he believes of the S-O bond rupture in APS reduction to sulfite and, like the theory of Reese, additional fractionation is likely a kinetic effect associated with one or more of the reductive steps in the pathway.

III. Carbon and Electron-Donating Substrates

Like other organotrophic microorganisms, Desulfovibrio sp. require organic carbon for cell synthesis, and as an electron donor for energy production (i.e. an oxidizable substrate). The fact that they obtain energy by anaerobic respiration of sulfate, sulfite, thiosulfate, etc., with electrons from an organic source makes them chemoorganotrophs as stated in the Bergey's Manual, 8th Ed. (1974).

Data on growth or sulfide yields, or both, on materials such as yeast extract or peptone (based on batch culture experiments) have often been reported (Bunker, 1939; Butlin, 1949; Macpherson, 1963). Postgate (1951) carrying out addition experiments, attributed the effects of yeast extract and peptone to their content of serine, ornithine, isoleucine, and cysteine. It was later found by Grossman (1953) that cysteine was merely fulfilling a poising role and could be replaced by Na_2S. Postgate (1965) reported, as well, that ornithine, serine and isoleucine could be replaced by EDTA, a chelating agent.

He suspects the amino acids merely render inorganic iron available in the presence of sulfide. Thus, to a large extent, the situation returns to the opinions held in the earlier literature, that inorganic iron partly accounts for the growth-stimulating effect of yeast extract; the other stimulants are unidentified and may not, in fact, be real in the nutritional sense.

The existing literature with respect to the nutrition of sulfate reducing microorganisms is essentially devoid of information concerning the role of a given compound as a carbon substrate and as a potential electron source (or in some cases as a potential electron acceptor). To this writer's knowledge, the only paper specifically dealing with the role of an organic substrate in the nutrition of Desulfovibrio sp., is that of Michalus and Rittenberg (1960). The following summarizes their findings using iso-butanol as the target substrate. Growth with this substrate, as measured by H_2S production or mg of $BaCO_3$ formed from wet combustion of cells, required yeast extract, and neither labelled CO_2 nor iso-butanol were incorporated into the cells appreciably. Iso-butanol was, however, stoichiometrically oxidized to iso-butyric acid. In fact, under these conditions, the bulk of the cell carbon apparently originated from the added yeast extract. Possibly the role that yeast extract plays in the carbon nutrition of these organisms depends on the growth conditions, and what other carbon substrates are available.

Lactate is the most common carbon and energy source used in media for growth of sulfate reducing bacteria. It is oxidized to acetate via the phosphoroclastic reaction, yet there has never been a full description of the first enzyme in the pathway, i.e. lactic dehydrogenase because of its apparent, extreme instability, LeGall and Postgate (1973). Since pyruvate is an intermediate in the oxidation of lactate to acetate and CO_2 then it too should satisfy the energy and carbon requirements of Desulfovibrio sp. When pyruvate is the primary source of electrons and carbon, certain species of Desulfovibrio can grow either in the presence of sulfate, using classical dissimilatory sulfate reduction to remove electrons, or in the absence of sulfate with hydrogen production acting as the electron sink (LeGall and Postgate, 1973). In the former case the organisms can use oxidative phosphorylation and substrate-level phosphorylation as a means of energy generation, in the latter they are forced to depend on substrate level phosphorylation as their sole means of energy metabolism. Akagi and Verna (1966) studied the phosphoroclastic reaction in crude cell-free extracts of D. vulgaris. They reported that the addition of ferredoxin alone was sufficient to couple pyruvate dehydrogenase to hydrogenase, but full activity was restored only with addition of both ferredoxin and cytochrome C_3.

In a brief but thought-provoking paper, Vosjan (1970) compared growth yields of D. desulfuricans with pyruvate and lactate as carbon sources. Under the conditions imposed in this study, at higher substrate concentrations, sulfate became the limiting factor and growth on lactate stopped, while growth on pyruvate continued, but at a slower rate. When sulfate was present, the cell yield/mole of substrate was 6.9 g for pyruvate and 4.9 g for lactate. After $SO_4^=$ exhaustion, these yields fell to 1.2 g and 0 g respectively. His conclusion, based on these results, was that electrons from pyruvate enter the electron-transfer chain at a lower redox potential than those from lactate. Electrons from pyruvate could enter the system at a E_o' as low as -700 mV, 280 mV below the H_2 formation reaction. Electrons resulting from lactate oxidation to pyruvate would enter an electron-carrying system at a potential no lower than -190 mV unless exogenous energy was provided. Vosjan also concluded, although no experimental proof was provided, that there might be a phosphorylation site between the point where the electrons from pyruvate and from lactate enter the ETS.

Postgate (1959) established that most Desulfovibrio sp. can dismute fumarate or malate without growth. Miller and Wakerley (1966) pointed out that D. gigas and several strains of D. desulfuricans could grow while dismuting fumarate.

The mechanisms of the fumarate dismutation pathway in D. gigas are summarized in the review article of LeGall and Postgate (1973). Fumarate is converted to malate by a fumarate hydratase with the malate being decarboxylated to pyruvate by a malate enzyme. There is no direct formation of lactate from malate. In addition, the malate enzyme is NADP specific. In the absence of sulfate, pyruvate can be carried through the phosphoroclastic reaction to yield acetyl phosphate and CO_2 and molecular hydrogen. The potential also exists here for the formation of 1 mole of ATP by substrate-level phosphorylation. In addition, a membrane-linked fumarate reductase has been found which can link pyruvate oxidation to the reduction of fumarate with the production of succinate. In the presence of $SO_4^=$, however, neither of these reactions is necessary since the oxidation of pyruvate is then linked to dissimilatory sulfate reduction.

IV. Carbon Dioxide Fixation and Mixotrophy in Desulfovibrio sp.

Carbon dioxide assimilation by Desulfovibrio sp. during heterotrophic growth is more pronounced than by other heterotrophs. Postgate (1960) published results showing that D. desulfuricans assimilated three times as much carbon dioxide as did Aerobacter aerogenes under similar growing conditions. In addition, Sorokin (1961) isolated an unclassified strain of Desulfovibrio which apparently assimilated 30% of its cell carbon from CO_2 fixation compared with 3-8% fixed by other heterotrophs. Presumably the balance of its carbon originated from conventional sources. Unfortunately the literature is devoid of publications indicating the actual biochemical reactions involved in CO_2 fixation in Desulfovibrio sp.

Desulfovibrio sp. also demonstrate the phenomenon of mixotrophic growth; i.e. the utilization of organic compounds for synthesis purposes with the aid of energy obtained by inorganic oxidations. The most common form of inorganic oxidation in these organisms centers around hydrogen oxidation.

It has been proven by the extensive work of Sorokin (1966) that a strain of D. desulfuricans isolated from the stratum water of an oil deposit was able to grow by oxidation of molecular hydrogen or formate. Apparently biosynthesis occurred in the presence of small amounts of acetate which together with carbon dioxide fully provided the carbon requirements of the organism.

Yagi, (1958, 1961) reported reversible oxidation of CO by sulfite in cell-free extracts of D. vulgaris was stimulated by the addition of formate. The reaction was essentially a generation of hydrogen and CO_2

from the reaction of CO with water.

V. Other Carbon Sources Utilized by D. desulfuricans (strain Hildenborough)

The Hildenborough strain of D. desulfuricans has been one of the most widely employed strains of Desulfovibrio in laboratory studies in the last fifteen years. This strain was purified by Postgate (1953), and is the only strain of Desulfovibrio whose range of carbon substrates has been systematically studied, MacPherson and Miller (1963). Their study included some forty alternative nutrients and electron donors. The basal medium used for maintaining the strain contained lactate as the only carbon source and electron donor. Utilization of a given substrate was measured by visually observing the cultures and scoring them after 5 successive transfers on their particular media on a scale from 1-4 (based on cell turbidity and H_2S production). For the alternate substrate study, lactate was removed and the various substrates were substituted. Lactate at equivalent concentration proved to be the best substrate for growth. Substrates giving significant amounts of growth were pyruvate, glycerol, sucrose and fructose. Substrates giving detectable growth within seven days incubation time included glucose, lactose, maltose, methanol, ethanol, propanol, oxaloacetate, glutamate, glutamine, asparagine, and yeast extract. Substrates which gave marginal growth and eventually led to the strain dying out upon transfer were fumarate, succinate, succinamide, L-aspartic acid, L-cysteine, L-histidine, DL-isoleucine, DL-methionine, L-phenylalanine, DL-threonine, DL-tryptophane and DL-valine. Twelve substrates tested could not support growth and included acetate, citrate, malate, DL-alanine, L-arginine, L-citrulline, glycince, L-leucine, L-lysine, L-proline and DL-serine. MacPherson and Miller also made mention of the fact that in a few instances, most noteably with glycerol, a "training" period (i.e. increasing glycerol concentrations slowly in the growth medium) was necessary before the organisms could successfully utilize the provided substrate.

As stated previously, this is one of the few examples of a purified isolate of Desulfovibrio being tested against a number of possible electron donors and carbon substrates, before studies on the characteristics or peculiarities of the organism's electron-transferring capabilities, or its particular dissimilatory sulfate reduction route were carried out. This is an unfortunate situation which has certainly hindered the full elucidation of the general biochemical capabilities of Desulfovibrio sp., Postgate (1965).

VI. The Utilization of Hydrocarbons by Dissimilatory Sulfate-Reducers and Their Role in Crude Petroleum Degradation

The question of the role of sulfate-reducers in the biogenesis of petroleum deposits has generated a point of contention between the microbiologist and the geochemist. In general, the geochemist accepts the hypothesis that apart from geological processes involved, petroleum genesis is partially dependent upon microbiological activities. An overall hypothesis was outlined by Hammar (1934) and is still accepted, for the most part, as being true. In essence, his theory was as follows. Aerobic bacteria initially degrade all or most of the oxygen-containing materials within organic deposits. This ultimately produces reducing conditions and a greatly lowered oxygen tension. This situation allows a population succession to occur, namely the stimulation of an anaerobic population. These anaerobes continue to metabolize and lower the oxygen, nitrogen and sulfur content of organic constituents. Lipid material such as sterols, fatty acids and waxes are enriched, ultimately yielding organic materials in the source sediments which more closely resemble petroleum. ZoBell (1944) pointed out the changes in elemental analysis during petroleum diagenesis. Carbon levels rose from 49% in marine sapropel to approximately 85% in petroleum. Comparison of the two extremes showed the oxygen content falling from 40% to less than 1%, and the nitrogen content decreasing from 5% to less than 0.5%. The hydrogen content increased from 5% to 12-13%. ZoBell went on to claim that all of these changes can be brought about in vitro using anaerobic Clostridium and Desulfovibrio species.

Opinions vary, however, between the geochemist and microbiologists, with respect to the role or activity of anaerobic organisms within deposits of geothermally mature petroleum. By using in vitro conditions of anaerobiosis, various microbiologists have obtained conflicting results as to utilization of n-alkanes as carbon substrates by anaerobic or facultative bacteria. Initial work by various research groups did show that almost without exception, sulfate-reducers could be isolated from oil field waters in producing areas, (David, 1967). Beck (1947) tested the ability of sulfate-reducers to grow and generate H_2S in a mineral salts medium with crude oil as a sole source of carbon. The systems were heavily inoculated with the cultures, thus small amounts of H_2S generated were discounted as being contaminant carry-over. Controls of cultures inoculated into salts media with lactate as carbon source nearly always yielded significantly more H_2S. Only three of all cultures tested showed any significant amounts of H_2S generated using petroleum as a carbon substrate. There is no indication from Beck's work that his cultures were completely free from non-sulfate reducing bacteria. Beck concluded that utilization of petroleum was at best very slow among sulfate-reducers.

Rosenfeld (1960) applied for a patent claiming the detection of sulfate reducing bacteria in subsurface waters as being an index of petroleum being present. This reasoning was based on an apparent belief of their hydrocarbon-utilizing ability. The method as listed in the patent does not, however, include hydrocarbon oxidation as a criterion but simply claims the detection of such microorganisms grown on a lactate-containing medium. Thus, in the opinion of subsequent workers in the field, the patent is of questionable value to the petroleum industry. Updegraff and Wren (1954) are of the opinion that when petroleum is used as a substrate for anaerobic growth, the utilization of certain non-hydrocarbon components might be the means of growth for sulfate-reducers. This would greatly depend on the chemical nature of the crude oil. The exact nature of such "non-hydrocarbon components" is certainly at this point, still conjecture.

Updegraff (1954) carried out exhaustive studies in an attempt to determine the ability of sulfate-reducers to use petroleum hydrocarbons as substrates for energy and growth. Of all the isolates they had at their disposal, they chose eleven for experimental work. Like other workers before them, they used hydrogen sulfide generation as evidence of bacterial activity and not increase in cell mass or utilization of the added hydrocarbon. The results indicated that 4 of 11 strains of sulfate-reducers were able to generate H_2S. This was taken as being indicative of hydrocarbon utilization. They stated but showed no proof that all their strains of sulfate-reducers were pure cultures.

The field research laboratories of the Socony Mobil Oil Company have in the past, carried out various research projects concerning the role of microorganisms in subsurface deposits. The results of a few of these projects have been reported by Davis (1967). This information was available to Davis, as he was a consultant in certain of the projects (private communication). In one of the projects, the oxidation of hydrocarbons by D. desulfuricans was studied by means of radioactive labelling. In view of what was known about this topic from past research experience, it was decided that to seriously consider a relationship between these bacteria and hydrocarbon utilization, with a subsequent production of hydrogen sulfide, certain limitations would have to be allowed. Thus under rather ideal experimental conditions, the oxidation of radioactive methane and ethane was tested. After incubation of their systems for approximately one month, both the carbon dioxide in the atmosphere above the plate cultures and the Desulfovibrio cells were radioactive indicating that both methane and ethane were utilized by the sulfate reducing bacteria.

This work is reasonably significant in any discussion of microbiology pertaining to the field of petroleum research, since it is evidence to indicate that sulfate-reducers play at least a small role in subsurface metabolism of volatile petroleum components. It is unfortunate, however, that the only description of the experiments appear in the textbook by Davis (1967) with no actual data being presented. Therefore it is impossible to judge how much label conversion was taken to be significant in the study.

Earlier work by Rosenfeld (1947), Uspenskii et al (1947), Shturm (1951), and Dostalek et al (1957) had also attempted to show positive utilization of petroleum hydrocarbons by sulfate reducing bacteria. Typically their methods depended upon the reduction of methylene blue as evidence of hydrocarbon oxidation. Such methods are now known to be insensitive and subject to errors because of uncontrolled variables. Thus their work cannot be accepted with as much confidence as can the work carried out by the field research labs of Socony Mobil Oil Company. However, the work of Uspenskii et al (1947) does show one interesting fact; namely that amounts of apparent hydrocarbon oxidation by sulfate-reducers were extremely dependent on the type of petroleum used. Generally oils of high paraffinic character were preferred substrates as opposed to oils of high naphthenic or asphaltic character.

Much of the research in the period 1940-1950 with respect to the role of sulfate-reducers in petroleum degradation was carried out by, or influenced by ZoBell (1944), (1947). It would appear that his group enriched many mixed anaerobic populations containing sulfate-reducers capable of utilizing non-hydrocarbon components as well as paraffinic hydrocarbons. As well ZoBell claimed to have isolated an organism, D. hydrocarbononoclasticus which in pure culture could anaerobically utilize paraffinic hydrocarbons as the sole energy and carbon source. However, several aspects of this work must be criticized. First of all, his claim of Desulfovibrio sp. degrading large quantities of paraffin material must be questioned since no direct chemical evidence of this was presented other than detection of low concentrations of fatty acids which could conceivably result from cell autolysis. His statements concerning the purity of his culture of D. hydrocarbonoclosticus cannot be substantiated since the strain has been lost and has never been re-isolated, nor was it ever submitted to a type culture collection. His use of ascorbic acid to poise his defined growth medium must be questioned, since in none of his work does he show adequate controls to determine whether his "purified" sulfate-reducer or his sulfate reducing mixtures could use ascorbate as an electron donor or carbon source. MacPherson and Miller (1963) state that caution should be observed in using ascorbate as a reducing agent in media for sulfate-reducers for this very reason. His work with admittedly mixed cultures of facultative anaerobes and sulfate-reducers which liberate bound petroleum from tar sands, glass, wool, ignited sand, etc., was impressive in that significant amounts of oil could apparently be released, judging by the photographic evidence presented in his papers, and in Davis (1967). The release of oil was apparently due to surface-active agents and CO_2 produced by the bacterial flora.

One of the most important contributions in terms of the ecology of sulfate-reducers in oil deposits was the work carried out by Kuznetsova and Gorlenko (1965). This work centered around the Romashkino oil field in the USSR, which has been maintained in a producing state by the commonly used procedure of water injection wells. After approximately one year, this fresh injection water appeared as co-produced water in the pumped oil. From this co-produced water, Kuznetsova and Gorlenko successfully isolated a mixture of microorganisms composed of sulfate-reducers, a Pseudomonas sp. and another unidentified facultative anaerobe. Experiments were then designed to duplicate growing conditions found within the oil deposit. The results of these experiments can be summarized as follows. Dissolved H_2S was evident in the co-produced waters, and in vitro, the most active formation of H_2S in a mineral medium with petroleum during the growth of the microorganism complex was at 27-30°. At 40° sulfate reduction was essentially absent. The optimal temperature for the growth of the sulfate-reducing bacterium from the organism complex was 40° while that of the Pseudomonas sp. was 27-30°.

Their data indicated that sulfate reduction, at the expense of oxidation of petroleum hydrocarbons was a complicated process in which not only sulfate reducing bacteria took part, but also the Pseudomonas sp., which probably carried out initial petroleum oxidation. Thus it would seem that sulfate-reducers indirectly use petroleum hydrocarbon components and as yet, their direct use of hydrocarbons in an anaerobic environment has not been conclusively proved.

Postgate (1960) also questioned whether there is sufficient sulfate in and around the average oil deposit to allow appreciable amounts of sulfate-reducer growth using petroleum hydrocarbons as substrate.

Although not indicated in Kuznetsova's paper, it is probable that the injection water supplies dissolved oxygen to the facultative portion of the mixed population, and hence the initial hydrocarbon oxidation is via a classical aerobic mechanism as proposed by Winters and Williams (1969), Jobson et al (1972) and Bailey et al (1973).

VII. Syntrophy Between Sulfate-Reducers and Other Bacteria

Sulfate reducing bacteria show a rather limited substrate specificity, so syntrophy with other bacteria is of considerable ecological importance. Tezuka (1963, 1964), after a long and complicated study of the Sumida River, showed that a lactic-acetic fermentation of carbohydrate-like material provided substrates for bacterial sulfate reduction. Organisms such as *E. coli* and *A. aerogenes* were implicated as being responsible for the initial fermentation. Tezuka could also show that crude enrichments of sulfate-reducers would utilize a wider variety of amino acids and other materials than could pure cultures of *Desulfovibrio*.

Tuttle and co-workers (1969) uncovered a fascinating syntrophic relationship existing between cellulose-utilizing microbial mixtures and species of *Desulfovibrio*. Acidic mine leachates, rich in sulfate and iron, were passing through a wood residue pile from a sawmill. Action of the cellulolytic organisms in the region of the sawdust pile provided carbon substrate for dissimilatory sulfate-reducers which in turn caused sulfide production and the precipitation of iron as FeS. The net result was removal of iron and sulfate from the stream and a neutralizing of the mine leachate waters. The sulfate-reducers functioned in mixed cultures at pH 3; while in pure culture, they could not tolerate a pH of less than 5.0.

VIII. Mechanism of Dissimilatory Sulfate Reduction

The sulfate reducing bacteria, classified in the genus *Desulfovibrio*, characteristically oxidize molecular hydrogen with the reduction of

5

sulfate to sulfide in the absence of air. There is also good evidence to support the claim that they carry out oxidative phosphorylation. This evidence comes from studies carried out with whole cells and cell-free preparations, Barton (1972). The utilization of sulfate as an inorganic electron acceptor is unique in that it requires ATP for the activation of sulfate in the form of adenylyl sulfate (APS), Ishimoto (1959), Peck (1959). Dissimilatory sulfate reduction differs from the other form of inorganic respiration commonly found in nature, namely nitrate reduction (denitrification). In this case nitrate is reduced to nitrite with no preliminary activation by ATP required. The nitrite thus formed can accept additional electrons and be further reduced to either NH_3, N_2O or N_2. In the case of NH_3 formation, the pathway is exactly the same whether the organism is using it as a means of generating biosynthetic ammonia from nitrate or whether the organism is using nitrate for "dissimilatory" purposes as an electron acceptor in the absence of oxygen, Campbell and Lees (1967).

The role of dissimilatory sulfate reduction is obviously an essential one in Desulfovibrio sp. since this is their primary method of respiration. However, the steps of the process are still not completely understood.

Most workers agree that sulfate functions as the terminal electron acceptor for the anaerobic respiration of Desulfovibrio sp. It is from this premise that the bulk of the research has proceeded. The form in which sulfate is reduced has been clearly established as being APS, as mentioned before. The enzyme catalyzing this reaction is termed ATP sulfurylase, Ishimoto (1959). Inorganic pyrophosphatase is required in all likelihood for the formation of significant amounts of APS, since it is known that any accumulation of pyrophosphate blocks further synthesis of APS. APS is directly reduced to AMP and sulfite by the enzyme APS reductase, an iron-containing flavoprotein with a molecular weight of approximately 220,000 (Peck, Deacon, and Davidson, 1965). This initial two-step reduction to sulfite requires two electrons per molecule and is the first known stage of sulfate acting as a terminal electron acceptor. Extracts of Desulfovibrio sp. lack APS kinase and do not form, reduce, or metabolize PAPS (an intermediary step following formation of APS in the assimilatory sulfate reduction route) (Lee, LeGall, and Peck, 1971). However, they do possess low levels of a biosynthetic sulfite reductase (Lee et al, 1971). The action of this reductase is reversible with oxygen as electron acceptor. In cell-free systems this results in the formation of a high energy sulfate bond which can be exchanged for phosphate in the presence of ADP sulfurylase. This is the only known instance of substrate level phosphorylation using inorganic reactants (Lee et al, 1971; Robbins and Lippmann, 1958). This same system appears to be the mechanism involved in the oxidation of sulfur compounds in certain species of Thiobacillus (Bowen, (1966).

The route of dissimilatory sulfite reduction is different from the assimilatory pathway. It is now known to contain or involve at least three distinct enzymes and inorganic intermediates. The elucidation of this pathway has been classic in terms of elegance of experimental design (Suh and Akagi, 1966). They were the first to clearly demonstrate the accumulation of thiosulfate during sulfite reduction. Kobayashi, Tachibana and Ishimoto (1969) also presented convincing evidence that trithionate and thiosulfate were sequentially accumulated during the reduction of sulfite by fractionated extracts of D. vulgaris. The latter authors proposed the following series of reactions for the reduction of sulfite to sulfide:

$$3HSO_3^- + 3H^+ + 2e^- \xrightarrow{*} S_3O_6^{--} + 3H_2O \qquad [1]$$

*: Bisulfite reductase (desulfoviridin)

$$S_3O_6^{--} + 2e^- \xrightarrow{**} S_2O_3^{--} + SO_3^{--} \qquad [2]$$

**: Trithionate reductase

$$S_2O_3^{--} + 2e^- \xrightarrow{***} S^{--} + SO_3^{--} \qquad [3]$$

***: Thiosulfate reductase

Very firm evidence presented by Lee, LeGall, and Peck (1973) indicates that the actual species initially reduced in this sequence is bisulfite (reaction [1]). The enzyme involved, bisulfite reductase, corresponds perfectly with the properties of desulfoviridin and the two names are now considered synonymous (for this particular species). The enzymes involved in reactions [2] and [3], trithionate reductase and thiosulfate reductase respectively, have both been demonstrated in crude cell extracts. However, only thiosulfate reductase has been purified to any extent.

If one studies the paper by Barton and LeGall (1972), their proposal for the route of dissimilatory sulfate reduction is very similar:

$$ATP + SO_4^{--} \longrightarrow APS + PP_i \qquad [1]$$

$$H_2 + APS \longrightarrow SO_3^{--} + AMP + 2H^+ \qquad [2]$$

$$4H^+ + H_2 + 3SO_3^{--} \longrightarrow S_3O_6^{--} + 3H_2O \qquad [3]$$

$$H_2 + S_3O_6^{--} \longrightarrow S_2O_3^{--} + SO_3^{--} + 2H^+ \qquad [4]$$

$$H_2 + S_2O_3^{--} \longrightarrow S^{--} + SO_3^{--} + 2H^+ \qquad [5]$$

The only difference in this series of equations is in the origin of electrons for the system. Kobayashi and co-workers (1969) assumed an organic electron source, whereas the scheme of Barton and LeGall clearly demonstrates the feasibility of H_2 as an electron donor and illustrates four possible positions in the pathway that H_2 can enter the pathway. This would yield 8 electrons, sufficient to reduce one atom of sulfur from an oxidation state of +6 to -2.

The route of sulfate reduction to sulfide is reasonably well understood; the mechanism by which the electrons involved become an integral part of oxidative phosphorylation is, however, less than clear. Two basic problems exist which block our complete understanding of the energetics of these microorganisms. The first issue centers around the number of phosphorylations involved in reduction of sulfate with electrons from hydrogen or an organic electron donor. The $\Delta G'_o$ for the oxidation of four hydrogen molecules with the reduction of one molecule of sulfate to sulfide is -38.6 Kcal/mole; Barton (1972), or -35 Kcal, if one uses tables available in most physical chemistry texts. If one assumes an effeciency of about 50-60% (Stouthamer, 1973), in most bacterial systems there is sufficient energy for the formation of roughly three high energy phosphate bonds. This assumes the free energy of formation of ATP is -7.3 Kcal/mole (at 30° and optimal Mg^{++} concentrations). There exists three enzymatic reaction in the dissimilatory reduction of sulfite to sulfide (as mentioned previously) to which phosphorylation might be coupled. The $\Delta G'_o$ for the final reduction of thiosulfate to sulfide with hydrogen is -2.7 Kcal/mole; therefore this reaction cannot be linked to ATP formulation on thermodynamic grounds. Thus the only way 3 ATP's could possibly be generated would be the reduction of 3 sulfite molecules with the formation of one trithionate molecule, the reduction of trithionate to yield sulfite and thiosulfate and recycling of the two sulfites from trithionate and thiosulfate breakdown back into formation of trithionate.

Interestingly enough, the $\Delta G'_o$ for the oxidation of 2 moles of lactate to acetate with the reduction of one mole of sulfate is -40.6 Kcal/mole of sulfate. The oxidation of four moles of pyruvate in the same mechanism has a potential $\Delta G'_o$ of -85.2 Kcal/mole (Decker et al, 1970).

A second problem which complicates the matter further is the presence in Desulfovibrio sp. of several electron-transfer components which apparently link the electron donors (hydrogen or organic compounds such as lactate) with the primary acceptor (sulfate and further reduced intermediates).

D. desulfuricans was the first non-photosynthetic anaerobe in which the presence of a cytochrome, cytochrome C_3, was demonstrated, Ishimoto et al (1956). This cytochrome (E'_o = -213 mV) possesses a demonstrable heme prosthetic group and serves as an electron acceptor for hydrogenase, and a donor for sulfate reduction. Its molecular weight has been established as being 12,000 daltons (Postgate, 1956).

A second cytochrome, cytochrome b has been recently described by Hatchikian and LeGall (1972). It serves as an electron acceptor for hydrogenase and will donate to fumarate.

Hatchikian et al (1971) describe two additional cytochrome species. The first, cytochrome C-553, is smaller than cytochrome C_3 with a molecular weight of 9,000. It too possesses a heme prosthetic group and serves as the electron acceptor for formic dehydrogenase; however, no compound has been identified to which it can donate electrons. The second cytochrome described has been termed cytochrome cc-3 which possesses a molecular weight of 26,000 and of course a heme prosthetic group. It has not yet been determined as to which reactions donate electrons to this cytochrome. It can, however, serve as an electron donor for thiosulfate reduction.

Ferredoxin was suspected to be present in Desulfovibrio sp. by many researchers, but LeGall and Dragoni (1966) were the first to actually isolate it successfully from D. gigas. It has a molecular weight of 6500 daltons and possesses 4 iron atoms per molecule. It has been established that this ferredoxin serves as the electron acceptor for cytochrome C_3 and can donate electrons to sulfate reduction. Initial sequencing of this ferredoxin indicates a high degree of homology to clostridial ferredoxin (Travis et al, 1971).

An electron-carrying component called flavodoxin has also been isolated from various Desulfovibrio sp. Its molecular weight has been estimated to be 16,000 daltons; its prosthetic group being FMN. It is known that flavodoxin serves as an electron acceptor for reduced cytochrome C_3 and will successfully donate to sulfate reduction (LeGall et al, 1967).

One electron carrier has been isolated from D. vulgaris which is known to accept electrons from $NADH_2$. This carrier, rubredoxin, having an estimated molecular weight of 6500, possesses a non-heme iron prosthetic group and can donate electrons to cytochrome C_3 (LeGall, 1968).

All of the above-mentioned electron carriers have been shown to be active in electron transfer; however, most of the observations have been made with crude or impure preparations. Because of this, it is not yet possible to assign discrete physiological roles to each carrier. Actually, the multiplicity of carriers has been a problem in attempting to define the roles of a specific carrier. The occurrence of some of the carriers in soluble protein is necessary for cell synthesis purposes, but may also be an artifact of cell breakage, since it is expected that if these carriers are involved in oxidative phosphorylation they should be located in the membrane fractions of Desulfovibrio sp. LeGall and Postgate, 1973).

Proof of oxidative phosphorylation coupled to the oxidation of hydrogen with sulfate in crude free extracts of Desulfovibrio sp. has been shown by Peck (1966), and, as mentioned previously, by Barton (1972). It is known that the energy released in the oxidation of hydrogen by sulfate is about -40 Kcal/mole of sulfate. As inferred previously, if the available energy was released equally in the four reductive steps of dissimilatory sulfate reduction, none of the steps would provide sufficient energy to support oxidative phosphorylation.

Therefore the occurrence of intermediates in the reduction of sulfite may represent an adaptation to restrict the major energy-releasing steps to permit phosphorylation coupled to electron transfer. Apparently this view is supported by the fact that the E'_o of thiosulfate reduction is close to that of the hydrogen electrode and that the E'_o of the trithionate/thiosulfate couple is around +0.2 mV (Peck, 1974). Peck further believes that electron transfer may occur from specific electron donors, such as lactate, through specific electron transfer chains to specific acceptors such as a trithionate reductase.

IX. Hydrogen Metabolism in Desulfovibrio Species and Other Associated Organisms

In the introductory remarks of this literature review there was brief mention made of the production of hydrogen by Desulfovibrio sp. in the absence of sulfate. This is apparently limited to situations when simple substrates such as pyruvate and perhaps ethanol are the primary electron donor. In most cases, however, sulfate is available to Desulfovibrio sp. in nature, or is provided in laboratory experiments. Thus these organisms are able to use H_2 as an electron donor with sulfate as the acceptor. It would appear that this is one of the most overlooked phenomena in nature as the following statements will attempt to indicate.

Postgate (1960) in his review article concerning the economic activities of sulfate reducing bacteria indicates that practically any environment which either produces hydrogen or has iron-water interaction can support these organisms. This assumes of course the availability of a suitable carbon substrate(s). This opinion is also expressed by Davis (1967), Peck (1974), Roy and Trudinger (1970) and Trudinger (private communication, 1974). It is generally accepted that sulfate-reducers play a major role in anaerobic corrosion processes. They do this chiefly by shifting reaction equilibria (i.e. removal of hydrogen from iron-water interfacial areas). This reaction was termed cathodic depolarization, in that dissimilatory sulfate-reducers possess hydrogenase with which they depolarize the surface of wet metal by removing the protective film of cathode hydrogen.

Interpretation of corrosion coupled with cathodic depolarization by Roy and Trudinger (1970) is as follows:

1. $4\ Fe \rightarrow 4\ Fe^{++} + 8\ e^{-}$ (anodic reaction)
2. $$\left.\begin{array}{l} 8\ H_2O \rightarrow 8\ H^{+} + 8\ OH^{-} \\ 8\ H^{+} + 8\ e^{-} \rightarrow 4\ H_2 \end{array}\right\}\ \text{(cathodic reaction)}$$
3. $SO_4^{--} + 4\ H_2 \rightarrow S^{--} + 4\ H_2O$ (cathodic depolarization - Desulfovibrio)
4. $Fe^{++} + S^{--} \rightarrow FeS$ (corrosion product)
5. $3\ Fe^{++} + 6\ OH^{-} \rightarrow 3\ Fe(OH)_2$ (corrosion product)

Simply on the basis of these five reactions, dissimilatory sulfate-reducers constitute a very influential microbiological group in terms of their environmental effect.

Peck reported in his review article on the evolutionary significance of inorganic sulfur metabolism (1974), that hydrogenase activity could be washed from certain D. gigas isolates by vortexing in phosphate buffer and assaying the wash according to the method of LeGall et al (1971). Using procedures employed to demonstrate the periplasmic location of enzymes, it was possible to remove 90% of the hydrogenase from D. gigas.

Hydrogenases in various microorganisms have been classified according to their molecular weight, solubility, and susceptibility to aggregation, Kidman (1969). Their work indicated that hydrogenase from D. gigas had a molecular weight of 56,000 daltons and was composed of an undetermined number of distinct molecular species. It is generally believed that hydrogenase (E_o' of -410 to -420 mV) activity is directly coupled to ferredoxin (-413 mV) or flavodoxin which in turn can donate to cytochrome c_3 (-203 mV) present in Desulfovibrio sp. Electrons at the level of cytochrome c_3 then enter the dissimilatory sulfate reduction pathway.

X. Interspecies Hydrogen Transfer

As basic studies have progressed in the area of microbiological relationships within the sulfur cycle, it has become apparent that microbial types such as the purple sulfur bacteria can derive their electrons from H_2S production by sulfate-reducers. In some cases there may also be a transfer of reducing power from certain photosynthetic bacteria to sulfate-reducers. A growing body of evidence is being assembled which suggests that such transfers do occur (Bryant, 1969), and that the molecule actually transferred is molecular hydrogen. Further, growth of D. vulgaris and D. desulfuricans on pyruvate in the absence of the obligatory terminal electron acceptor sulfate has been observed when these organisms are cultured with the H organism isolated from Methanobacillus omelianskii. Methane is formed presumably the the utilization of the electrons derived from the oxidation of pyruvate, Bryant (1969). From these and other observations concerning altered fermentation in mixed population, Wolin and Bryant have developed the concept of interspecies hydrogen transfer.

XI. Growth Yield Studies of Desulfovibrio sp.

Desulfovibrio sp. occupy a unique position in anaerobic microbiology due to their abilities to carry out oxidative phosphorylation. Yet as stated by Barton et al (1972) in their paper dealing with oxidative phosphorylation in Desulfovibrio, it is not at all clear how much ATP is in fact generated in a given round of dissimilatory sulfate reduction. Thus to speak of Y_{ATP} values with respect to Desulfovibrio sp. without a clear picture of their energetics is, to say the least, suspect. The use of the term Y_{ATP} in discussing cellular metabolism dates back to the pioneering work of Bauchop and Elsden (1960). They measured growth yields of several microorganisms in terms of grams of cells formed per mole of ATP (hence Y_{ATP}). These determinations were made with organisms and growth conditions in which the net ATP gain per mole of substrate was accurately known from the metabolic balances and studies in vitro on the enzymic pathways. It is also important to note that all calculations were made on cultures growing in complex media which contained high concentrations of amino acids or yeast extract. Under these conditions, practically none of the cellular carbon was derived from the substrate (e.g. glucose) which was exclusively utilized as an energy source. From these measurements Bauchop and Eldson established a Y_{ATP} mean value of 10.5 g (dry weight) of cells formed per mole of ATP. The measurements which yielded this mean varied from 8.3 to 11.5 g cells/mole of ATP. Since the publication of this paper, most physiologists believed that the value of 10.5 represented a constant in microbial metabolism.

Senez (1962) grew the Canet strain of D. desulfuricans on a defined medium containing excess sulfate as electron acceptor and NH_4Cl as the nitrogen source with either pyruvate or lactate as carbon and energy source. He obtained molecular growth yields which gave an average of 9.6 grams (dry wt.) of cells/mole of substrate utilized. This he rounded to 10 grams for convenience and then reasoned that since the accepted Y_{ATP} value was 10.5, this strain of Desulfovibrio must be gaining a net of about one mole of ATP/mole of lactate or pyruvate consumed. He then went on to propose a pathway of dissimilatory sulfate reduction. In this scheme, electrons came from either lactate (4 electrons) or pyruvate (2 electrons) and were accepted by cytochrome C_3. His scheme showed the transfer step from lactate or pyruvate to cytochrome C_3 as producing one ATP/pair of electrons transferred. This ATP was then utilized in the scheme in that 0.5 moles ATP were consumed in the activation of 1/4 SO_4 and the remaining 0.5 moles ATP were consumed in the reduction of 1/4 SO_3 to sulfide. Thus oxidative phosphorylation yielded no net ATP. The one net ATP/mole of substrate consumed came from the substrate level phosphorylation occurring in the conversion of acetyl phosphate to acetate. As stated before, this whole metabolic scheme was proposed with no proof other than its serving as an explanation of how D. desulfuricans could have a Y_{ATP} value close to that reported earlier by Bauchop and Elsden. The utilization of 0.5 moles of ATP in the reduction of 0.25 moles of sulfite to sulfide is very questionable, and has not been included in other dissimilatory sulfate reduction schemes published subsequent to 1962.

More recent work by Stouthamer and Bettenhaussen (1973), has cast severe doubt on the earlier belief that a Y_{ATP} value of 10.5 is more or less constant among different microorganisms. They believe that the value of 10.5 might be considered as an upper limit for cells of normal average composition growing in a medium where full energetic coupling occurs. They do indicate, however, that even higher yields can be demonstrated. In an experiment with known metabolic pathways and satisfactory carbon recoveries, it was proven that Lactobacillus casei had a Y_{ATP} value of approximately 20. On the other hand Zymomonas anaerobia gave a value of only 5.9. Stouthamer and Bettenhaussen argued in their paper, with convincing evidence, that Y_{ATP} for an organism is determined by the cell composition, the specific growth rate and the maintenance coefficient. Thus it would seem that the figure of 1 mole of net ATP generated by Desulfovibrio sp. from a mole of lactate or pyruvate, thus giving a Y_{ATP} value of approximately 10 cannot be fully accepted since the pathways laid out to explain these values are based on a constant Y_{ATP} value of 10.5 which may or may not always be correct. For this reason there is no reliable evidence to suggest what amount of net energy Desulfovibrio sp. gain from coupling lactate or pyruvate oxidation to dissimilatory sulfate reduction. Moreover, the degree of efficiency of oxidative phosphorylation in Desulfovibrio sp. is in question (Barton, 1972).

MATERIALS AND METHODS

I. Sources of Chemicals, Reagents, Solvents and Petroleum

All chemicals used were of reagent grade and were obtained from commercial sources. Solvents used for chromatography were of infra-red spectranalyzed quality, and were obtained from Fisher Scientific Company. Silica gel (28-200 mesh) and F-20 Alcoa Alumina gel (80-200 mesh) were purchased from Matheson, Coleman and Bell.

The supply of North Cantal petroleum used was obtained from Imperial Oil Co., Calgary, and originated in the Weyburn Oil Field, Province of Saskatchewan.

II. Microbiological

A. Cultures Employed

Most of the research carried out for this thesis involved studies on a pure culture of a Desulfovibrio species isolated from a sample of North Cantal oil. This isolate, provisionally identified as a strain of Desulfovibrio vulgaris, sub-species oxamicus, will be referred to as "Isolate #1."

Two mixed cultures, each containing Desulfovibrio spp. and facultative aerobes, were obtained by enrichment procedure using soil samples from Nipisi (Alberta) and Norman Wells (Northwest Territories). These mixed cultures will be referred to as MC #1 and #2 respectively.

B. Media

Butlin's medium (1949) was used for maintaining cultures, but was modified for continuous culture studies. The levels of $MgSO_4 \cdot 7H_2O$ were reduced and $FeSO_4$ and $CaCl_2 \cdot 6H_2O$ were omitted to minimize the development of precipitates. The composition of this medium, referred to as Medium #1, is as follows:

K_2HPO_4	0.5 g
NH_4Cl	1.0 g
Na_2SO_4	2.0 g
$MgSO_4 \cdot 7H_2O$	0.1 g
Sodium lactate (60%)	1.5 ml
Yeast extract (Difco)	1.0 g
Distilled water	1000.0 ml
pH after autoclaving	7.1 - 7.2

For nutritional and growth experiments, the levels of medium components were varied according to the following list:

Medium #2:	Sodium lactate:	4.5 ml/l
Medium #3:	Sodium lactate:	4.5 ml/l
	Na_2SO_4:	4.0 g/l
Medium #4:	Yeast extract:	3.0 g/l
	Na_2SO_4:	4.0 g/l
Medium #5:	Sodium lactate:	4.5 ml/l
	Yeast extract:	3.0 g/l
	Na_2SO_4:	4.0 g/l
Medium #6:	Sodium lactate:	4.5 ml/l
	Yeast extract:	2.0 g/l
Medium #7:	Yeast extract:	omitted
Medium #8:	Yeast extract:	omitted
	Sodium lactate:	omitted

A modification of Brewer's medium was employed (Collins, 1967) for viable count studies. Dextrose was omitted and sodium lactate, sodium sulfate and ferrous sulfate were added, resulting in a medium of the following composition:

Meat extract	1.0 g
Yeast extract (Difco)	2.0 g
Peptone (Difco)	5.0 g
Sodium lactate (60%)	2.5 ml
Na_2SO_4	2.0 g
Sodium thioglycollate	1.0 g
$FeSO_4$	0.1 g
Agar	15.0 g
Distilled water	1000.0 ml
pH adjusted to	7.0 - 7.2

In an experimental series involving oil catabolism, a medium developed by ZoBell (1947) was modified by substituting 1.0 ml of North Cantal crude petroleum for sodium lactate and distilled water for sea water:

K_2HPO_4	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Na_2SO_4	1.0 g
Na_2SO_3	0.2 g
$CaCO_3$	0.2 g
NH_4Cl	0.1 g
Ascorbic acid	0.1 g
Sodium lactate (60%)	3.0 ml
Sea water	1000.0 ml
pH	7.0 - 7.5

C. Culture Maintenance

Capped tubes (18 x 150 mm) containing 15 ml of Medium #1 were used for growing and maintaining cultures of Isolate #1 and the mixed populations. The addition of two small, sterile, iron finishing nails to each tube resulted in the potential being poised in the bottom of the tubes to levels as low as -280 mV (Pankhurst, 1971). This technique was used as a convenient method of developing and maintaining anaerobic conditions. Quantities of culture could be removed by the use of sterile Pasteur pipettes from the area around the nails and transferred to a tube of fresh medium containing poising nails. In this way, stock cultures were easily maintained and transferred in an active state.

D. Enumeration of Sulfate-Reducers

The assay of soil and water samples for the presence of sulfate-reducers was carried out using a modified most probable number procedure. Dilutions of the sample were made in milk dilution bottles containing 3 mM phosphate buffer (pH 6.5), poised with 0.1% sodium thioglycollate. One milliliter of such dilutions were added to 18 x 150 mm tubes containing 10 ml of normal Butlin's medium plus two iron finishing nails. Groups of five tubes were inoculated with aliquots of each dilution. These tubes were briefly mixed and then incubated at 30°C for 14 days. At the end of the incubation period, the tubes were scored for positive blackening (indicative of FeS production as a result of H_2S liberation). The results of the scoring were compared with most probable number tables to obtain the "most probable viable count".

Viable count studies on cultures of Isolate #1 were undertaken in a different manner. The initial culture dilutions were prepared using 9 ml tube dilution blanks containing 3 mM phosphate buffer (pH 6.5) poised with 0.1% sodium thioglycollate. One-tenth ml volumes of these dilutions were added to triplicate tubes containing 10 ml of Brewer's agar held at about 43°C. These inoculated Brewer's tubes (screw-capped) were inverted four times and allowed to harden. The caps were loosened and then the tubes placed in an anaerobic jar (BBL). The jar was charged with a gas-pak, sealed, and incubated at 30° for four days. On the fourth day, the tubes were removed and black colonies within the agar counted in those tubes which gave sufficient dilution to allow the recognition of distinct colonies. This procedure usually limited the counting to tubes showing less than seventy colonies per tube. Incubation for less than four days did not allow for full colonial growth at higher dilutions. If left for more than four days, most positive tubes would show blackening throughout, so that individual colonies could not be recognized.

E. Electron Microscopy

Samples for electron microscopy were supported on carbon-shadowed, Formvar-coated, copper grids. These preparations were negative-contrast stained with 3% (W/V) phosphotungstic acid and examined in a Phillips Model 200 electron microscope.

III. Culturing Techniques

A. Batch Culture

Various volumes of both Isolate #1 and the mixed cultures were grown under batch culture conditions. Regardless of the medium being used, the following protocol was adhered to. The media, vessels and a supply of 10% sodium thioglycollate (in distilled water) were autoclaved separately. When cool, media were added aseptically to the growth containers and sufficient thioglycollate solution was added to to yield a final concentration of 0.1% (W/V). As soon as was possible, the media were inoculated using 48-72 hour tube cultures. Depending on what the culture was to be used for, inoculation rates varied from 5 to 15% (V/V). The inoculum was transferred with sterile Pasteur pipettes by expelling it at the bottom of the vessel containing the fresh medium. The inoculated cultures were then incubated (usually 30°) until sufficient growth had occurred as measured by visual estimation or optical density increase.

B. Continuous Culture

The chemostat used was that of Dawson (1963) which was designed for cultivating aerobic organisms. For this reason, certain modifications in protocol were necessary to cultivate an anaerobic microorganism. Culture volumes were maintained at 2.2 or 2.3 liters rather than 500 ml as used by Dawson. This aided in the development and maintenance of an anaerobic environment. Nitrogen gas, rather than air, was continuously sparged through a chemostat at a rate of 12 liters/hour. This served 4 purposes:

1. a positive pressure was generated within the chemostat and helped maintain sterility;
2. the manometric liquid level control was operated;
3. reducing conditions in the chemostat chamber were maintained;
4. metabolic H_2S was flushed out of the system to prevent toxic concentrations from accumulating (a major problem in batch culture systems).

The procedure for inoculating and establishing a culture of Isolate #1 in this chemostat was as follows. The pre-sterilized chemostat unit was filled to the required volume with sterile medium. Sufficient sterile sodium thioglycollate solution was added to yield a final concentration of 0.05% (w/v). Following this chemical poising, the medium was inoculated with 50-60 ml of 48 hour culture. The unit was then circulated with no influent or effluent flow permitted for a 24 hour period. This period allowed initiation of growth to occur with sufficient H_2S being generated so the system became self-poising with respect to reduction potential. Influent medium could then be pumped into the system thus creating a functioning chemostat. The chemostat was monitored for contamination by aerobic and anaerobic plating before and during experimental runs.

IV. Analytical Methods

A. Cellular Analyses

(i) Cell Fractionation

The procedure used was a modification of the Schmidt-Thannhauser technique (1945). The method is based on the sensitivity of RNA and the resistance of DNA to alkaline hydrolysis. The procedure used is summarized as follows:

One hundred ml volumes of batch- or chemostat - grown culture were centrifuged at 10,000 x g for 20 minutes at 2°. The cell pellet(s) was washed 3 times in 10 ml volumes of cold 3 mM phosphate buffer, pH 6.5. The pellets were then suspended in 10 ml volumes of the same buffer and duplicate 10, 50 and 100 µl volumes were removed for protein analyses. The remaining 9.68 ml of cell suspension were centrifuged to recover the cells. The pellets were suspended in 10 ml volumes of 0.2 N $HClO_4$ and centrifuged at 27,000 x g for 20 minutes at 2°C. The 0.2 N $HClO_4$ wash containing the acid-soluble pool extracted from the cells was stored for analysis if labelled-uptake studies were involved. The cell pellets were hydrolyzed by suspension in 0.3 N NaOH and incubated for 60 minutes at 37°. The DNA and protein present in the hydrolyzed samples were precipitated by addition of 2.5 ml of pre-chilled 1.2 N $HClO_4$. The suspensions were centrifuged for 20 minutes (27,000 x g), and the supernatants (6.5 ml) were saved for RNA analyses. The precipitates were washed with an additional 2.5 ml volume of 1.2 N $HClO_4$, centrifuged, and the supernatant combined with the previous volume to give a total of 9.0 ml of solution for RNA analyses. The pellets were solubilized by the addition of 2.0 ml of 0.2 N NaOH and these volumes were used to assay for DNA. Original culture supernatants (100 ml) were frozen and stored for lactate and sulfate determinations.

(ii) Protein

The washed cell suspension aliquots (10, 50, 100 μl), taken for protein analyses were hydrolyzed by the procedure of Herbert, Phipps and Strange (1971). The protein contents of hydrolyzed samples were determined by the procedure of Lowry et al. (1951), with the exception that color development was read at 750 nm rather than at 650 nm. A standard curve, using bovine serum albumin, was included in every assay.

(iii) RNA

RNA determinations were based on the standard orcinol reaction for ribose (Ashwell, 1957). A standard curve using known D(-) ribose concentrations was included in every assay. Since this assay only detects purine ribose, sample values were multiplied by 2.0 to give an estimate of total RNA-ribose. An estimate of total RNA was made by multiplying the total ribose content by 2.28 (weight conversion from the sugar to the average nucleotide weight).

(iv) DNA

DNA determinations were made using the diphenylamine reaction for deoxy-d-ribose (Ashwell, 1957). As with RNA determinations, a standard curve using deoxy-d-ribose was included in each assay. Values obtained were multiplied by 2.0 to give an estimate of total deoxy-d-ribose in the sample. An estimation of total DNA present was made by multiplying the total deoxy-d-ribose content by 2.44 (conversion from the sugar to the mean nucleotide weight).

(v) DNA Isolation

The method of Marmur (1961) was used for DNA extraction with the exception that deproteinization was carried out only one additional time following T_2 ribonuclease treatment. This gave a DNA product sufficiently pure for buoyant density determinations. Dilutions of the preparation were scanned spectrophotometrically to ensure a minimum of absorbance at 280 nm, and a reasonably well-defined absorbance at 260 nm. The amount of extracted DNA was measured using the relationship of one absorbance unit at A_{260} being approximately equal to 50 μg/ml of DNA.

(vi) Percent Guanosine and Cytosine

The purity of DNA prepared from Isolate #1 was analyzed by a buoyant density centrifugation procedure in CsCl (Fisher) made up in distilled water to a refractive index of 1.3995 ($\rho = 1.699$). When accurate buoyant density measurements were required for the calculation of percent G + C content approximately 4 μg of Escherichia coli B DNA ($\rho = 1.710$) was added to 7.9 μg of Desulfovibrio DNA. A sample of this

CsCl-DNA solution was centrifuged at 44,000 rpm (25°) in a Beckman Model E ultracentrifuge for 22 hours to achieve equilibrium. Photographs of the nucleic acid band(s) were taken using UV optics and transposed to a traced form using a Beckman Analytrol film densitometer. On the basis of the measurements of these tracings and using standard formulae, the buoyant density of the Desulfovibrio DNA was determined. The percent G+C content of the DNA was calculated as described later in the text.

(vii) Lipid Extraction and Methylation

For these determinations, washed cells were resuspended in 5 ml of 0.1 N HCl and mixed until a uniform suspension had been achieved. Ten ml of 2:1 (v/v) chloroform-methanol was then added to these suspensions and mixed for 2 minutes. The emulsions formed were broken by centrifugation and 2 ml of the lower chloroform layers were carefully removed and evaporated to dryness under a stream of nitrogen. When dry, the samples were dissolved in 2 ml volumes of absolute methanol plus 2 drops of concentrated H_2SO_4, transferred to methylation flasks, and refluxed for two hours to achieve complete methylation. The flasks were cooled and the contents transferred to small separatory funnels. Transfer was completed by rinsing the flasks with 5 ml volumes of n-hexane. Three ml volumes of water were then added to each separatory funnel and the combined contents thoroughly shaken. The hexane (upper) layers were removed. The aqueous phases were re-extracted with 5 ml of hexane and the 2 hexane fractions were combined. The total hexane extracts were washed with 5 ml volumes of $KHCO_3$ (1 M) in order to neutralize any residual acids, and then washed with two 5 ml volumes of distilled water. The hexane volumes were transferred to screw-cap tubes and evaporated to dryness under nitrogen. The residues were re-dissolved in 50 µl volumes of methylene chloride and dried by adding small amounts of Na_2SO_4. The samples were then analyzed by gas chromatography.

(viii) Cytochrome Difference Spectrum

The method used to analyze the visible light absorption spectrum of whole cells was as described by Jones (1971, 1972). Sodium dithionite-reduced whole cell suspensions of Isolate No. 1 were blanked against oxygen-oxidized cell suspensions and scanned using a Pye Unicam (SP-8000) recording spectrophotometer. The scan was continued from 630 nm to 410 nm and all absorption bands within this region were re-scanned at least twice.

B. Chemical Analyses

(i) Sulfate

Sulfate concentrations were determined using a turbidimetric procedure. The reagent used was a product of Hach Chemical Co., Ames, Iowa, (Sulfa Ver IV Sulfate Reagent). Samples of media were added to 50 ml volumetric flasks to give sulfate concentrations in the range 10-100 µg/ml. Flasks were shaken for 3 minutes after adding 0.5 g of reagent. The turbidity of each sample was read in a 1 cm cuvette at a wavelength of 540 nm. A standard curve was included in each assay using sulfate concentrations of 10, 20, 30, 50 and 100 µg/ml.

(ii) Lactate

The procedure described by Neish (1952) was used to determine lactate concentrations. This method is based on the conversion of lactate to acetaldehyde which is reacted with p-hydroxydiphenyl (p-phenylphenol) to yield a blue color. The absorption of the product is measured at 570 nm. A standard curve was run for each assay using from 1 μg to 12-15 μg total lactic acid per reaction volume. In addition, test samples were initially treated with 25% $ZnSO_4$ and 2 g of $Ca(OH)_2$ + 20% $CaSO_4$ to remove any protein and pyruvate which would interfere with this procedure.

(iii) Iodimetric Determination of Sulfite, Sulfide and Thiosulfate

Sulfite, sulfide and thiosulfate in culture media were determined by the procedures of Nickless (1968). The hydrogen sulfide sparged during chemostat studies was also measured since this constituted a portion of the total sulfide produced in the system. The sulfide present in the effluent gas was trapped by passage through a Fischer-Milligan collection assembly containing 200 ml of 2% (w/v) zinc acetate solution. The sulfide recovered as zinc sulfide was determined gravimetrically and iodimetrically.

The iodine solutions used in all titrations were made up to approximately 0.04 M concentration. They were standardized indirectly against a primary standard $K_2Cr_2O_7$, (in concentrated H_2SO_4). The standardization protocol used was as recommended by Neish (1952). The iodine remaining after samples of culture had been added was determined by titration with 0.012 M thiosulfate.

In all cases, the actual volume titrated consisted of:

0.04 M iodine solution	5.0 ml
Glacial acetic acid	5.0 ml
Culture sample volume	5.0 ml (usually)
Distilled water	to 100.0 ml

Control titrations were also included where 5 ml of the uninoculated medium was used in place of the 5 ml of culture.

C. Isotope Fractionation

Mass spectrometric analyses of sulfur isotope ratios were carried out on samples from the chemostat studies. The samples were of 2 types:

1. influent media before they were pumped into the chemostat;
2. effluent media from the chemostat after the cells had been removed by centrifugation.

Samples of sulfate from the influent and effluent media were obtained by $BaCl_2$ precipitation. The precipitates were recovered by centrifugation and the pellets analyzed for $^{32}S/^{34}S$ content.

The procedures for sulfur reduction, burning to SO_2, and analyses by mass spectrometry were all based on techniques worked out by Dr. H.R. Krouse, Department of Physics, University of Calgary. The analyses reported here were carried out in his laboratories, as per Krouse et al (1967).

(i) Chemical Reduction of Samples

Samples of sulfate as $BaSO_4$ were reduced to H_2S by placing them in a distillation apparatus. Forty ml of reducing acid mixture (500 ml of concentrated hydriodic acid, 245 ml of 50% phosphoric acid and 816 ml of concentrated HCl) were added to this apparatus which was then heated for 2 hours. The H_2S produced was sparged into a 100 ml volume of cadmium acetate trapping solution. After trapping as CdS, a two-fold excess of 0.1 N silver nitrate was added to convert the sulfide to AgS. This mixture was heated for 60 minutes to complete precipitation. The AgS precipitate was filtered through Whatman filter paper and dried. The samples were scraped from the filter paper and mixed with a ten-fold excess by weight of 1:1 $CuO:Cu_2O$.

This mixture was packed in quartz glass sections of tubing and the ends packed with glass wool.

(ii) Sample Burning

Each individual packed tube was placed in a furnace linked to a vacuum rack and burned at 1050° for 30-45 minutes to convert the sulfide to SO_2. The SO_2 produced was collected into break seal tubes by freezing in alcohol: dry ice and then pentane: dry ice. This enabled the CO_2 to be removed by evacuation, leaving pure SO_2.

(iii) Mass Spectrometry

Break seal tubes were sealed onto a vacuum rack connected to the mass spectrometer. The mass spectrometer used was a 12-inch, 90° magnetic analyzer equipped for the simultaneous collection and analysis of ion current of masses 64 and 66. The ion currents were amplified by vibrating reed electrometers and a solid state amplifier. The ratio of the output voltages from the vibrating reed electrometer and the amplifier were periodically displayed with a five-figure integrating digital voltmeter-ratiometer and printed. By using four magnetically-controlled valves, the flow of gas into the mass spectrometer could be switched instantly from a standard reference sample of SO_2 to the unknown sample being analyzed.

In the text, the mass spectrometer analyses are reported as per mil deviations of the $^{34}S/^{32}S$ ratio of a sample from the standard. Ten $^{34}S/^{32}S$ ratios were printed alternately for the unknown and standard, and six sets of these sample-unknown ratios were obtained during each run. The mean ratio was calculated for each set and the overall ratio of the printed ratios of the unknown and standard were calculated on a time-corrected basis.

The overall relationship was calculated as follows:

$$\text{uncorrected } \delta o/oo = \left(\frac{{}^{34}S/{}^{32}S \text{ unknown}}{{}^{34}S/{}^{32}S \text{ standard}} - 1\right) \times 1000$$

The uncorrected value could be corrected for the oxygen isotope effect (mass 64: ${}^{32}S-{}^{16}O-{}^{16}O$; mass 66: ${}^{32}S-{}^{16}O-{}^{18}O$) by multiplying the δ value by whatever correction factor applied.

V. ${}^{14}C$ Radioactive Techniques

A. Reaction Vessel

Radioactive studies were carried out in 250 ml screw-top culture flasks fitted with a center well which would hold 5 ml of KOH (20%) for trapping any CO_2 produced. In addition, a serum-stoppered fitting was annealed into the wall of each flask to allow injection of acid for culture acidification when total labelled CO_2 production was determined. The usual procedure used was to sterilize media and flasks separately. These were cooled and the requisite amount of labelled compound was added to the flask. If the label was in a solvent system, this was volatilized using sterile, filtered nitrogen gas. Growth media were then added to the flasks and the center well was carefully greased with Vaseline. Five ml of 20% KOH were then pipetted into the center well(s). Finally, the flasks were inoculated with culture, sealed, and incubated at 30°C. After incubation, the flasks were normally injected with 10 ml of one molar phosphate buffer, pH 6, to lower the pH below neutrality. The flasks were then slowly agitated for a further 2 hours at 37° to allow CO_2 release and absorption by the KOH. The flasks were then opened, the KOH removed and the cells recovered by centrifugation for macromolecular ${}^{14}C$ analyses.

In the case of labelled studies using C-1 labelled lactate, major amounts of $^{14}CO_2$ were produced. Therefore, in order to ensure maximum transfer of CO_2 into the KOH, duplicate cultures were set up and incubated. When the experiment was terminated, one set of flasks was acidified with sulfuric acid while the other set was kept for cellular analyses.

B. Labelled Substrates

(i) Lactate

^{14}C-1-labelled lactate (specific activity 4.53 mCi/m mol; 98% radiochemical purity) was purchased as the sodium salt from New England Nuclear.

(ii) Yeast Extract

Uniformly-labelled yeast protein hydrolysate (specific activity 0.96 mCi/mg protein) was purchased from ICN Pharmaceuticals, Inc. This hydrolysate was used without further treatment.

(iii) Stearic Acid

^{14}C-1-labelled stearic acid was purchased from Amersham/Searle (specific activity 56 mCi/m mol; 98% radiochemical purity).

(iv) Octadecane

Mono-terminally ^{14}C-labelled octadecane (specific activity 21 mCi/m mol; 99% radiochemical purity) was purchased from Amersham/Searle.

C. Scintillation Counting

The radioactivity in the $^{14}CO_2$ trapped in the KOH was accomplished by spotting 50 μl volumes on uniform pieces of Whatman paper, drying under a heat lamp and counting in toluene-based scintillation fluid (10 ml/vial). Duplicate samples were counted for 10 minutes using a Nuclear Chicago Mark I scintillation counter. Radioactivity was calculated and included a correction for a quench factor obtained by counting labelled lactate spotted with and without 50 or 200 µl amounts of KOH suspended in the toluene fluid.

In counting fractionated components obtained from the Schmidt-Thannhauser procedure, all perchloric acid washes or extracts were neutralized to pH 7 using KOH. The potassium perchlorate was removed by centrifugation and sample volumes of up to 200 µl were counted in Bray's scintillation fluid (10 ml/vial). DNA-protein pellets from the Schmidt-Thannhauser procedure were resuspended in distilled water and 50-100 µl volumes were counted in duplicate in Bray's scintillation fluid (10 ml/vial).

VI. Chromatographic Techniques

A. Thin Layer Chromatography

(i) Lactate

The purity of the ^{14}C-labelled lactate used in the uptake studies was checked by chromatography on silica gel G plates. Two solvent systems, ethanol-ammonium hydroxide-water (80:4:16) and n-butanol-formic acid-water (10:1:5), were employed. The chromatograms were developed in ascending fashion at least 10 cm beyond the origin.

(ii) Stearic Acid

Both ^{14}C-labelled stearic acid and cell fractionation products containing labelled stearic acid were analyzed by thin layer chromatography. Samples were spotted on silica gel G plates and were chromatographed in hexane-ether-acetic acid (90:10:1).

(iii) Octadecane

Mono-terminally-^{14}C-labelled octadecane and cell fractionation products containing labelled octadecane were analyzed using thin layer chromatography. Samples spotted on silica gel G plates were chromatographed in hexane-ether-acetic acid (9:10:1).

Quantitation of label distribution for (i), (ii) and (iii) involved scraping the thin layer chromatograms in 1 cm portions and counting in toluene-based counting fluid. This value was compared to the activity present in the original sample.

B. Liquid Chromatography of Crude Petroleum

The liquid chromatographic fractionation of crude petroleum samples extracted from cultures was as described by Jobson et al (1972).

Oils were n-pentane-extracted from cultures, and also benzene-extracted when oil was applied in silica gel. The asphaltenic components were removed by passage through celite columns packed in pentane. The benzene-soluble asphaltenes and benzene-insoluble asphaltenes were subsequently eluted from the celite column with benzene, and 1:1 benzene:methanol respectively. De-asphaltened oil was further split into group fractions by layering on the top of dual-phase silica gel-alumina gel columns in normal pentane. Group fractions were eluted in order with n-pentane, benzene, and benzene:methanol (1:1), and yielded saturate, aromatic and NSO fractions respectively. By this liquid chromatographic procedure, a gravimetric group analysis of a petroleum sample before or after bacterial contact could be obtained.

C. Gas Chromatography

(i) Fatty Acid Methyl Esters

Gas chromatographic separation of fatty acid methyl esters was achieved using a 6 ft x 6 mm (I.D.) glass column packed with Chromasorb P-AW (100/120 mesh) which had been coated with 15% ethylene glycol succinate. A Hewlett Packard 7200 series gas chromatograph fitted with hydrogen flame detectors was used for all analyses under the following conditions:

Injector temperature	250°C
Column temperature	190°C (isothermal)
Detector temperature	250°C
Carrier gas: (N_2)	60 ml/min
H_2	39 ml/min
Air	240 ml/min

Peak areas were determined using a Hewlett Packard electronic integrator. A composite sample of known fatty acids (as methyl esters) was run three times to accurately determine retention times and to aid in the identification of extracted fatty acid components. The peak responses from injections of a known amount of methylated heptadecanoic acid were used to quantitate the identified fatty acids as per Ettre and Kabot (1963).

(ii) Petroleum Saturates

Gas chromatographic separation of saturate components was carried out using a 20 ft x 0.125 inch (O.D.) stainless steel column packed with Chromasorb W(60/80 mesh) coated with 5% OV-101. A Varion 1700 series gas chromatograph fitted with hydrogen flame detectors was used under the following conditions:

Injector temperature	295°
Column temperature	50-300° @ 10°/min (program)
Detector temperature	300°
Carrier gas (N_2)	15 ml/min
H_2	25 ml/min
Air	300 ml/min

RESULTS AND DISCUSSION

I. Physiological Characteristics of an Unidentified, Purified, Sulfate-Reducing Isolate

A. Enrichment of the Organism from North Cantal Oil

Initially, bacterial enrichments were set up using capped 18 x 150 mm test tubes as the enrichment chambers. These tubes contained 15 ml of Medium #1, two iron nails and quantities of North Cantal oil varying from 0.1 to 1.0 ml. The tubes were mixed carefully for approximately two minutes and then incubated at 22° and 30° for periods of up to 36 days. None of the tubes showed any blackening of the poising nails which would have been considered primary evidence of dissimilatory sulfate reduction. It was decided that this experimental design was weak in that the tubes were capped but not sealed, therefore allowing atmospheric air-exchange. The inclusion of methylene blue in uninoculated enrichment tubes showed that after autoclaving, the methylene blue was reduced to within 1 cm of the medium surface. The reduction of methylene blue elsewhere in the tube was maintained, presumably by the poising action of the nails. However, the top, unreduced region corresponded to the region where the North Cantal oil rose following initial mixing. It was probable that most, if not all, the microbial cells were still associated with the petroleum layer. Since this region was aerobic, it was unlikely that continued attempts to enrich an anaerobe using this procedure would have been successful.

It was decided, therefore, to switch to a flask enrichment procedure. The experimental design used allowed for development of a sealed anaerobic environment, and facilitated constant mixing between the oil, microorganisms, and the growth medium. This was achieved by using sterile 250 ml screw-top Erlenmeyer flasks filled to within 5-10 ml of the total capacity with sterile Medium #1. A 2 gm portion of sterilized iron wool was placed in the flask along with a sterile magnetic stirring bar which would fit into the cap of the Erlenmeyer, and finally, 1 ml of North Cantal oil was added. The cap was screwed in place and the whole assembly was fixed on a magnetic stirrer in the inverted position. (Plate I). When operating, the action of the magnet and iron wool created a vortex action which mixed the oil and the growth medium. After approximately 72 hr of incubation, blackening of the iron wool and medium took place, indicating the occurrence of dissimilatory sulfate reduction. (Plate I, photograph B). This enrichment was attempted four times on two different lots of North Cantal oil and positive growth was obtained within 80 hr in all four cases. When samples of this enriched culture were transferred in Butlin's medium, growth was apparent within 8 hr. Cultures could then be routinely transferred and maintained in an actively-growing state using the tube culture system. Cultures of this dissimilatory sulfate-reducing organism have been maintained for 2 1/2 years by routine transfer of culture to fresh tubes every 3 to 5 days. Initial attempts to preserve this culture by lyophilization have so far been unsuccessful.

The North Cantal oil used for the enrichments was obtained from the wellhead in Saskatchewan and was taken under aseptic conditions. The well was pumped for several days before the samples were taken in an attempt to flush contamination away from pumping parts in contact with

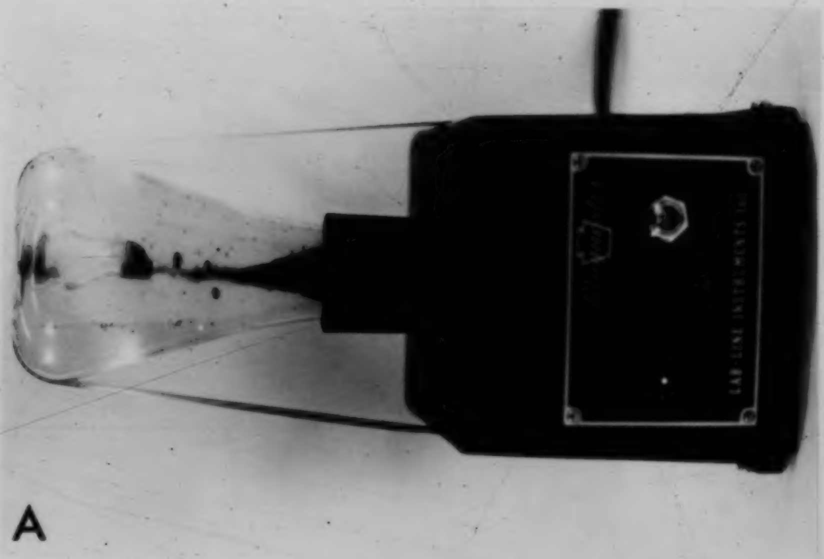
Plate I

EXPERIMENTAL DESIGN USED TO ENRICH SULFATE REDUCING ORGANISMS FROM NORTH CANTAL PETROLEUM

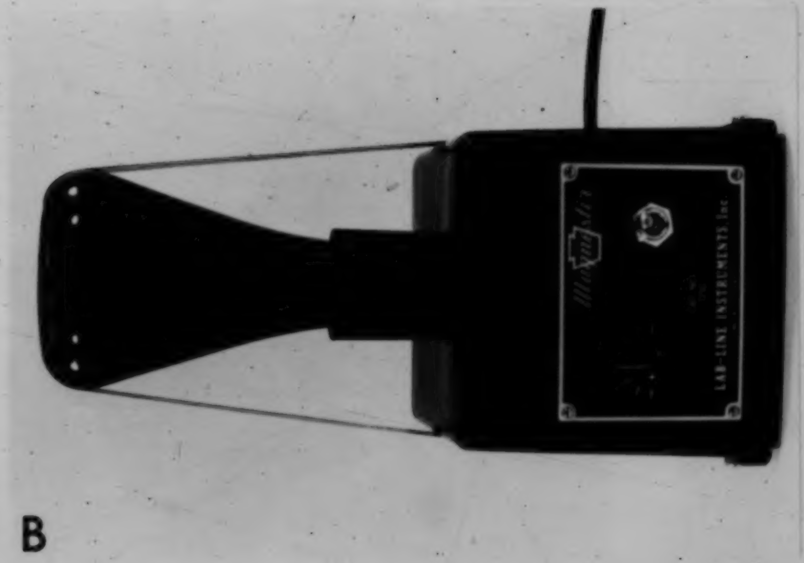
A. Inverted flask enrichment apparatus showing mixing action achieved under anaerobic conditions.

Incubation flask volume:	250 ml
Contents:	240 ml Medium #1
Incubation temperature:	35-39°
Volume of North Cantal oil:	1.0 ml

B. The inverted flask enrichment apparatus after approximately 80 hours incubation, showing evidence of dissimilatory sulfate reduction.


LAB-LINE INSTRUMENTS Inc.

A


LAB-LINE INSTRUMENTS Inc.

B

the oil. The oil was transferred using steamed hose to new steam-sterilized barrels. Samples of this oil were stored either in autoclaved ground glass-stoppered bottles, or in sealed sterile storage cans. Because of the stringent precautions imposed during sampling, it is unlikely the sulfate-reducing isolate resulted from the introduction of an incidental contaminant.

Examination of the culture by phase contrast microscopy showed the presence of vigorously motile, vibrio-shaped cells.

B. Determination of the Purity of Isolate #1

Cultures of both Desulfovibrio and Desulfotomaculum species are well-known for their tendencies to exist as mixed cultures containing both aerobic and facultative organisms (Butlin et al, 1949). For this reason, the newly-enriched culture from North Cantal oil was carefully studied to ensure that, in fact, it was axenic. Further phase contrast microscopy, as well as Gram-staining, culture on TYA, plate count, or blood agar failed to reveal the presence of any other aerobic or facultative organisms. Examination of diluted cultures growing in Brewer's agar tubes revealed only the presence of black, uniform, colonies after 96 hours at 30°.

Examples of electron microscopy of negatively-stained preparations of Isolate # 1 are presented in Plates II(a) and II(b). The cells are curved and possess a single polar flagellum. They range in size from 0.26 µm to 0.60 µm in diameter and up to 4.5 µm in length. Based on those observations, Isolate #1 appeared to be axenic.

C. Buoyant Density Measurements of DNA Extracted from Isolate #1

DNA was extracted from two cultures of Isolate #1 according to the procedure of Marmur (1961). The inoculum for one culture came from

Plate II(a)

ELECTRON MICROGRAPHS OF NEGATIVELY-STAINED

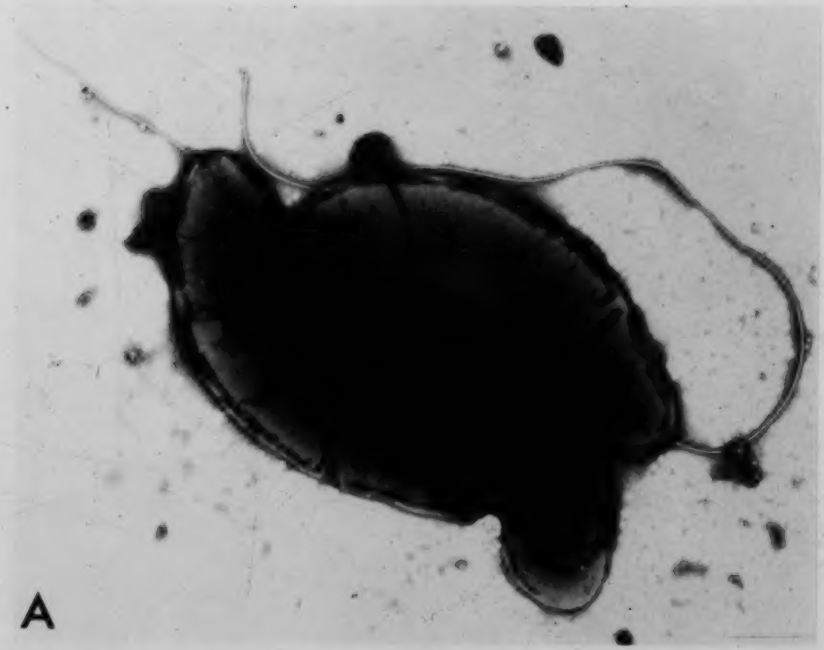
PREPARATIONS OF ISOLATE #1

A. Cells grown in continuous culture at an intermediate dilution rate (D = 0.095 hr^{-1}).

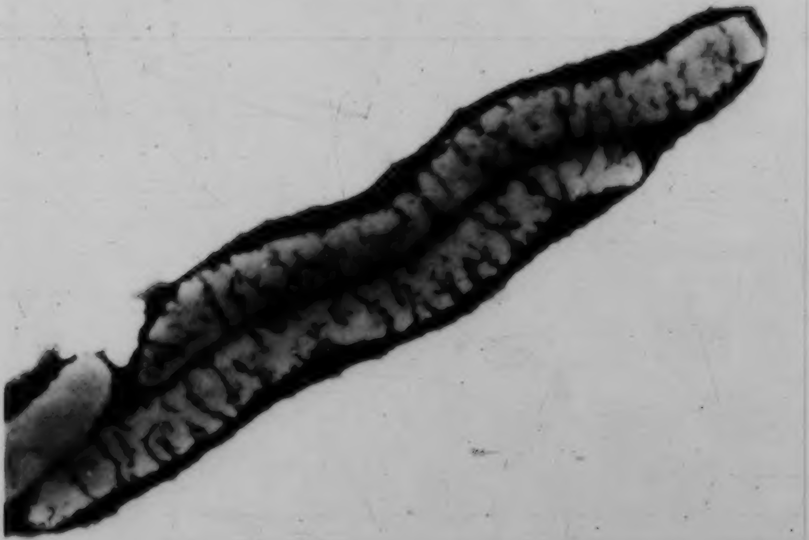
Magnification: x 26,200 (i.e., 1 μm = 2.62 cm)

B. Cells grown in continuous culture at an extremely low dilution rate (D = 0.012 hr^{-1})

Magnification: x 26,200 (i.e.. 1 μm = 2.62 cm)



A



B

Plate II(b)

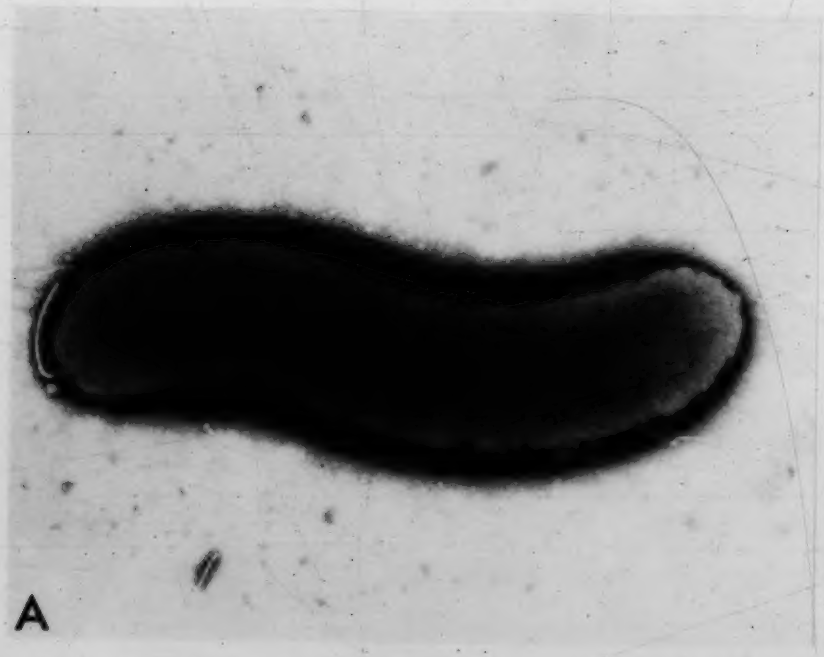
ELECTRON MICROGRAPHS OF NEGATIVELY-STAINED PREPARATIONS OF ISOLATE #1

A. Typical vibrio shape seen in cultures grown in maintenance tubes.

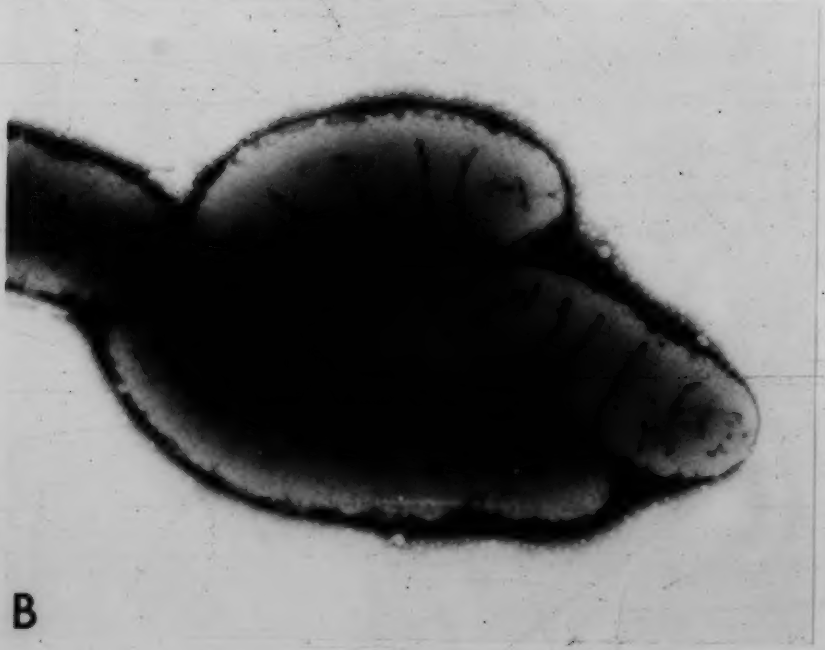
Magnification: x 46,750 (i.e., 1 μm = 4.67 cm)

B. Typical cell association seen in cultures grown in maintenance tubes.

Magnification: x 44,850 (i.e., 1 μm = 4.48 cm)



A



B

maintenance tubes of the organism while the second inoculum came from a continuous culture which had been operating for six months. Both samples of DNA, with added Escherichia coli B DNA as a marker (ρ = 1.710) were subjected to buoyant density centrifugation in CsCl. Densitometer tracings from both runs as well as values for buoyant densities of the two DNA samples are presented in Figure 1. The mean value for mole percent guanosine plus cytosine (G+C) content of Isolate #1 was calculated to be 65%.

D. The Relationship of the Percent Guanosine + Cytosine Content of Isolate #1 to Known Desulfovibrio Species

Attempting to classify Isolate #1 according to the scheme proposed by Postgate and Campbell (1966) and included in Bergey's Manual (8th edition) proved difficult. The scheme places all Desulfovibrio sp. into three groups based on their percent G+C content: 46(±1), 55(±1), and 61(±1). Using either the formula of de Lay (1970) or Szybalski (1971), Isolate #1, possessing a DNA buoyant density value of 1.7247, has a % G+C content of approximately 65(±1)%. It would thus appear that Isolate #1 might be a new species of Desulfovibrio since the established grouping varies by only 1%. However, a review of the earlier work by Saunders, Postgate, and Campbell (1964) showed that they used the calculation of Sueoka (1961) to establish their three classes of Desulfovibrio species.

The calculations presented in Table I show the three groupings of Desulfovibrio sp. as listed in the paper of Saunders et al (1964), the buoyant density values they obtained, and the resulting % G+C content they calculated using Sueoka's formula. The % G+C content values are also presented for these groups if the formulae of Schildkraut et al

Figure 1

DENSITOMETER TRACINGS AND BUOYANT DENSITY VALUES OF DNA FROM ISOLATE #1 AS DETERMINED BY EQUILIBRIUM CENTRIFUGATION

DNA samples from two preparations of the Desulfovibrio isolate were centrifuged with an E. coli B DNA marker in CsCl ($\rho = 1.710$) for 22 hr at 25° in a Model E ultracentrifuge. Ultraviolet scans of the cells were carried out when equilibrium had been achieved. These scans were converted to densitometer tracings from which the buoyant density of the Desulfovibrio DNA was determined by comparison with the marker DNA.

A. Densitometer tracing of an equilibrium run using DNA isolated from a maintenance culture of Isolate #1. Calculated buoyant density: 1.7248.

B. Densitometer tracing of an equilibrium run using DNA isolated from a culture of Isolate #1 grown in continuous culture for approximately 6 months. Calculated buoyant density: 1.7246.

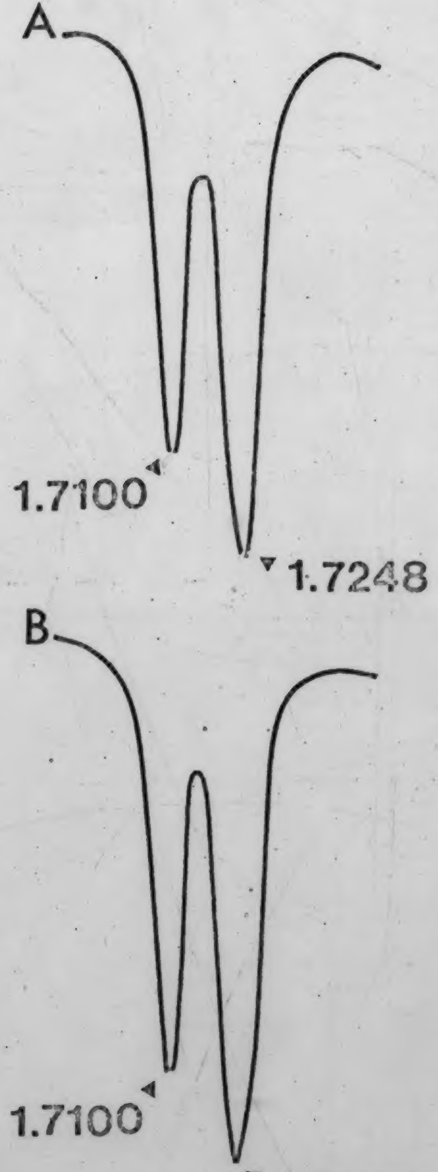

A
1.7100
1.7248
B
1.7100
1.7246

TABLE I. PERCENT G+C CONTENT VALUES OF MEMBERS OF GENUS *DESULFOVIBRIO* AS CALCULATED FROM BUOYANT DENSITY MEASUREMENTS USING DIFFERENT FORMULAE

Group (ρ)	Sueoka (1961)	Schildkraut et al (1962)	de Lay (1970)	Szybalski (1971)
I (a) 1.726	62.1	67.3	66.7	66.0
(b) 1.725	61.2	66.3	65.7	65.0
(c) 1.724	60.2	65.3	64.7	64.0
II (a) 1.720	56.3	61.2	60.6	60.0
(b) 1.719	55.3	60.2	59.6	59.0
(c) 1.718	54.4	59.2	58.5	58.0
III (a) 1.710	46.6	51.0	50.4	50.0
(b) 1.709	45.6	50.0	49.3	49.0
Isolate #1 av. 1.7247	60.8	66.02	65.4	64.7

Legend for formula: (ρ = buoyant density; P = % G+C)
Sueoka (1961): $\rho = 0.0103P + 1.662$ (used by Saunders et al, 1964)
Schildkraut et al (1962): $P = (\rho - 1.660)/0.00098$
deLay (1970): $P = 1020.6 (\rho - 1.6606)$
Szybalski (1971): $P = 1000 (\rho - 1.660)$

NOTE: Group I corresponds to *D. vulgaris*, *D. africanus*, *D. gigas*.
Group II corresponds to *D. desulfuricans*.
Group III corresponds to *D. salexigens*.

(1962) de Lay (1970), or Szybalski (1971) had been used. The values for Isolate #1 are also included under a separate category for comparative purposes.

It is apparent that Isolate #1 fits into Group I which consists of D. vulgaris, D. africanus, and D. gigas. Isolate #1 is unlike the latter two species in that it only possesses a single polar flagellum and is not lophotrichous. It also has been determined that the organism produces some transferrable growth on Butlin's medium in the absence of sulfate and with pyruvate substituted for lactate (i.e., sulfate-free growth on pyruvate). This precludes its classification as a typical strain of D. vulgaris since that species does not carry out sulfate-free growth. However, it is likely that the isolate is an example of D. vulgaris, subspecies oxamicus, which has identical characteristics to that of D. vulgaris but can also carry out sulfate-free growth on pyruvate.

The data in Table I suggest that all calculations of % G+C contents in Desulfovibrio sp. or for any bacterial genus should be based on a uniformly accepted formula. It is submitted in this thesis that the formula should not be that of Sueoka (1961) for the following reasons. This formula gives a % G+C content for E. coli of 45-46% based on the commonly accepted buoyant density of 1.710; whereas chemical analysis has placed the G+C content at 50%. This value is well within the range obtained by using the formulae of Schildkraut (1962), de Lay (1970), or Szybalski (1971). Moreover, the formula of Sueoka seems to be unquoted in modern literature except for the work of Postgate and Campbell.

E. Effect of Temperature on the Respiration of Isolate #1

The eighth edition of Bergey's Manual (Buchanan and Williams, 1974) states that, to date, the maximum temperature for growth of Desulfovibrio species is 44°. In order to compare this observation with Isolate #1 used in this study, an experiment was carried out whereby respiration at seven different temperatures was compared.

Figure 2 shows graphically the production of sulfide as a function of temperature. The production of hydrogen sulfide increased rapidly between incubation temperatures of 4° and 20°, and reached a maximum between 24° and 37°. The highest incubation temperature at which any significant amount of H_2S was produced was 42°.

It is to be realized that this experiment measured only the product of respiration (H_2S) and not growth (e.g., protein production). However, since energy production (respiration) is necessary for growth, within limits, H_2S production should have increased as the amount of growth by Isolate #1 increased. Hence, the measurement of respiration end-product (H_2S) after a fixed time period should have reflected the amount of growth which occurred.

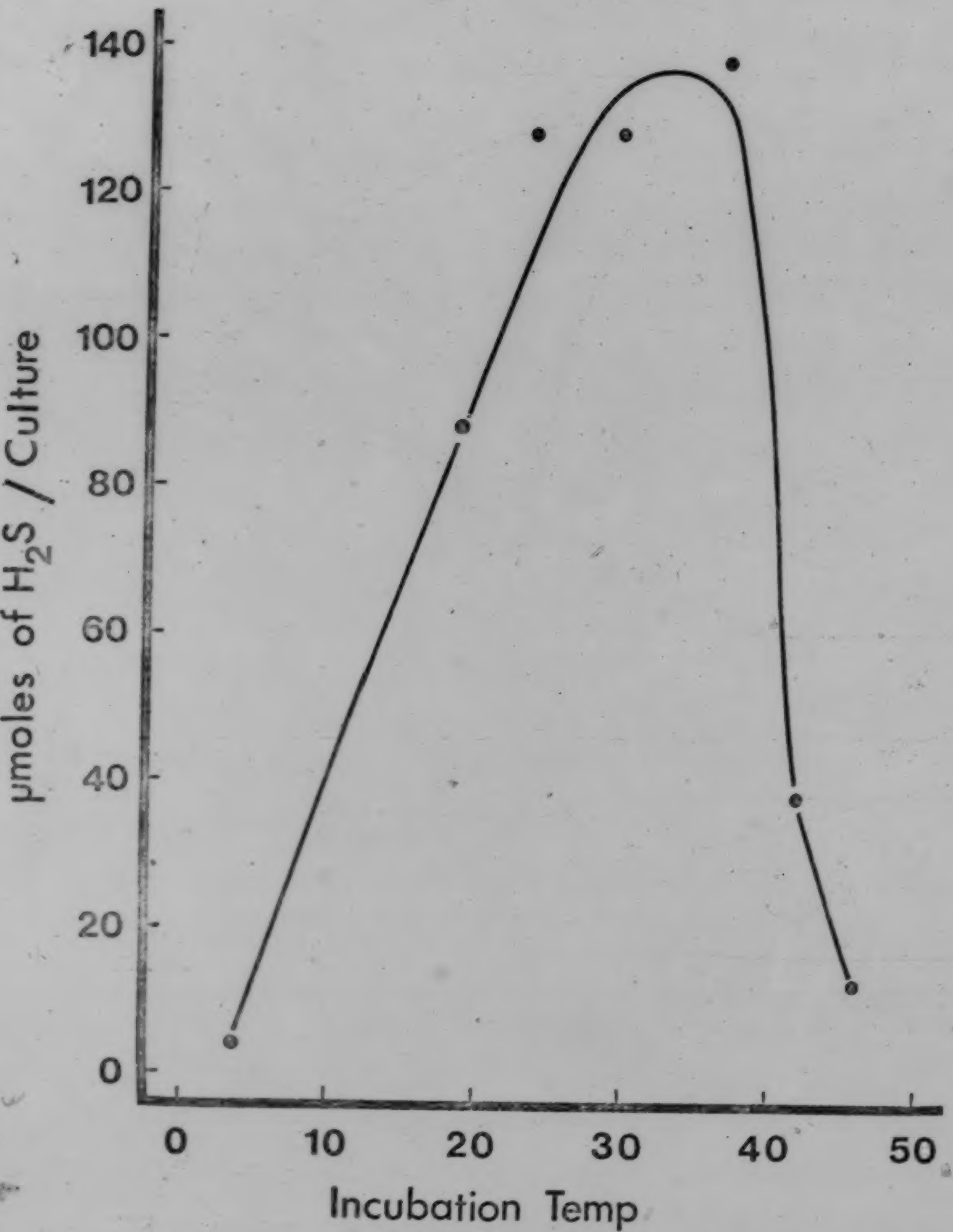
F. Determination of the Cytochrome Spectrum of Isolate #1

In dissimilatory sulfate-reducing bacteria, cytochrome C_3 and desulfoviridin have been considered characteristic pigments of the non-sporulating Desulfovibrio species (Postgate, 1956). Characteristically, difference spectra should contain predominant peaks in the 550-553 nm, 523-524 nm, and 420-422 nm regions if a C-type cytochrome is present. A spectrum, as shown in Figure 3, was obtained for Isolate #1 and appears to be that typically found in Desulfovibrio species containing a C-type cytochrome with α, β, γ absorption maxima at 552, 522, and 422 nm

Figure 2

HYDROGEN SULFIDE PRODUCTION OF ISOLATE #1
AS A FUNCTION OF INCUBATION TEMPERATURE

Incubations were carried out in 15 ml volumes of Medium #5 in sealed Hungate tubes. Each tube received 2 ml of-unwashed inoculum. Seven incubation temperatures were tested using duplicate incubation mixtures. Total incubation time was 14 days for all temperatures. Hydrogen sulfide produced was precipitated by the injection of 2 ml volumes of 2% zinc acetate, followed by vigorous shaking for 2 min. The zinc sulfide from each tube was centrifuged and the pellets thus obtained were subjected to iodimetry as per Materials and Methods.



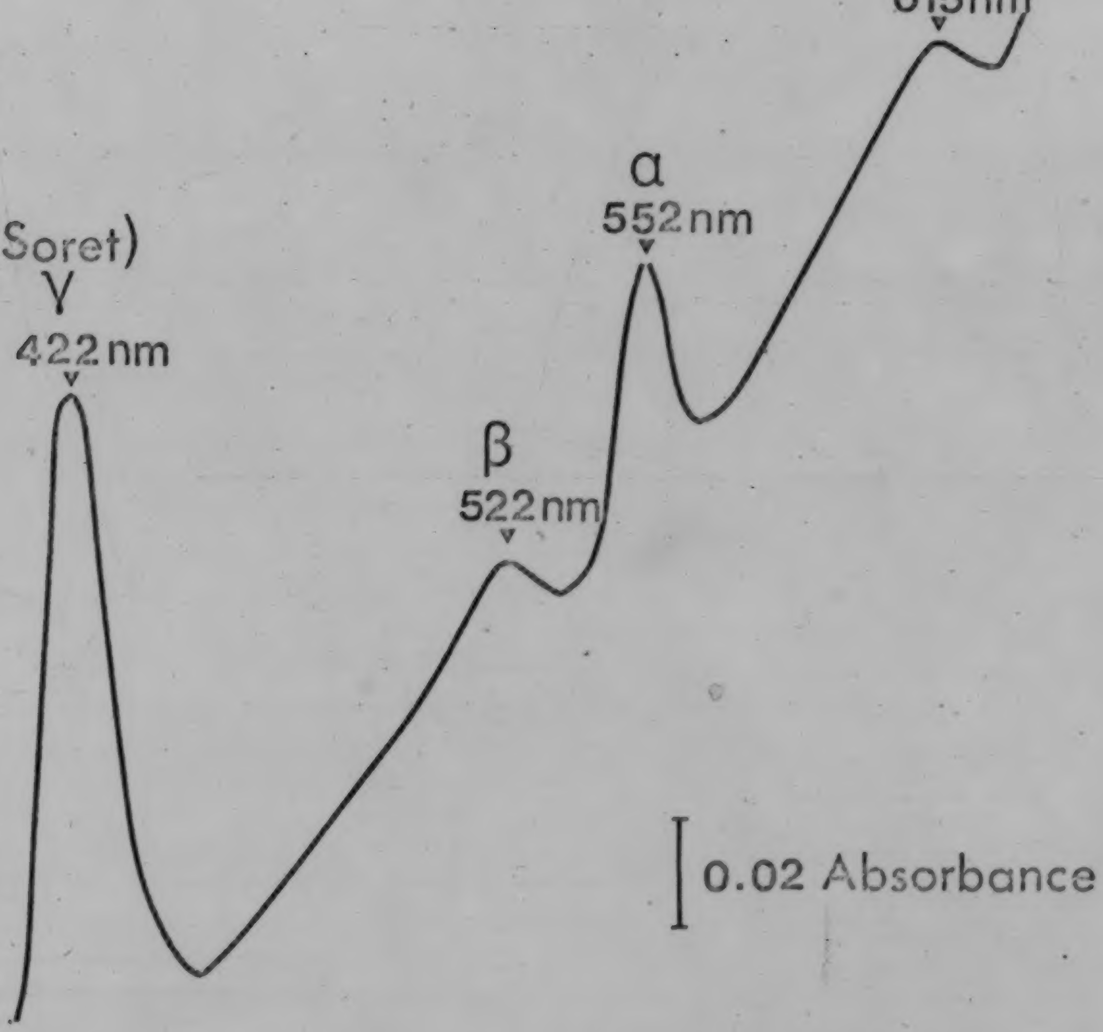
140
120
100
80
60
40
20
0
μmoles of H_2S / Culture
0
10
20
30
40
50
Incubation Temp

Figure 3

REDUCED MINUS OXIDIZED DIFFERENCE SPECTRUM
OF A WHOLE CELL PREPARATION OF ISOLATE #1

The overall procedure was as described by Jones (1972).

7


(Soret)
γ
422 nm
β
522 nm
α
552 nm
615 nm
0.02 Absorbance

respectively (422 nm corresponds to the Soret region). Apparently the absorption band found at 615 nm represents either a α peak of another cytochrome or of desulfoviridin.

Whole cell preparations of Isolate #1 in 0.2 N NaOH gave a very strong red fluorescence when excited with light at approximately 365 nm. This is indicative of the presence of desulfoviridin.

G. Studies Concerning the Response of Isolate #1 to Alternate Electron Donors

This study was undertaken to determine the ability of Isolate #1 to use substrates other than lactate. Medium #1 was the basal medium employed in the study with alternative electron donors replacing lactate to a final concentration of 10 mM. In order to ensure that growth actually took place, cultures on each electron donor were transferred five times at five-day intervals prior to the determination of amount of sulfide produced as a function of carbon source. After incubation of the fifth set of tubes for 5 days and the fourth set a total of 10 days, sulfide levels were determined iodimetrically. The presence of sulfide concentrations significantly higher than that in the basal medium control (Medium #1 - lactate) were deemed positive in terms of electron donor utilization and growth. Growth was inferred, since the ability to show significant sulfate reduction in five transfers indicated that the cells overcame five ten-fold dilutions and still reduced significant amounts of sulfate. The alternate electron donors which yielded either increased or similar levels of H_2S to the basal control are listed in Table II.

As in the study by MacPherson and Miller (1963), the presence of lactate as the primary electron donor resulted in the greatest production of H_2S. When used as the only electron donor and carbon source (Basal

TABLE II. SUMMARY OF AMOUNTS OF H_2S PRODUCED AS A FUNCTION OF ALTERNATE ELECTRON DONOR USED AFTER 5- AND 10-DAY INCUBATION PERIODS

Medium	Micromoles of H_2S Produced/Culture 5 Days Incubation	 10 Days Incubation
Basal + lactate	40.4	54.6
Basal + n-propanol	32.7	43.2
Basal + ethanol	25.6	36.0
Basal + oxaloacetate	22.7	35.6
Basal + pyruvate	13.7	27.5
**Basal + lactate (minus) yeast extract	22.8	24.9
Basal + citrate	10.2	15.7
Basal + malate	8.5	11.6
Basal + n-butanol	3.2	10.4
*Basal	7.4	7.5
Basal + stearic acid	4.6	7.5
Basal + acetate	8.8	7.1
Basal + asparagine	7.4	7.1
Basal + asparatate	4.6	7.1
Basal + palmitic acid	4.6	7.1

Basal Medium = Medium No. 1 minus lactate

*This served as the overall test control. All electron-donor candidates listed after this control gave similar or lower H_2S production values.

**This medium, and the H_2S production which occurred, indicates that yeast extract is not required, but is stimulatory, for the growth of this organism.

minus yeast extract), lactate yielded about 50% of the levels of H_2S that were detected in the presence of yeast extract (Basal). Because this isolate grew successfully through five transfers with lactate as the only electron donor and carbon substrate, it is obvious that it had no obligatory requirement for yeast extract. However, it is equally evident that factors within yeast extract were stimulatory to respiration and perhaps growth itself.

Table II clearly demonstrates that Isolate #1, like previously-described Desulfovibrio sp., can only make use of a relatively small variety of alternate electron donors. Propanol, ethanol, oxaloacetate, pyruvate, citrate, malate, and butanol were the only compounds tested which demonstrated any ability to replace lactate as an electron donor. All other compounds listed in Table II yielded responses not significantly different from growth on the basal medium itself.

Three amino acids (glutamate, alamine, glycine), three TCA intermediates α-keto glutarate, fumarate, succinate), two volatile fatty acids (propionic, butyric), glycerol, and six sugars (sucrose, galactose, lactose, maltose, glucose, fructose) were also tested as alternate electron donors. In all 15 growth trials, H_2S production was completely absent after the fifth transfer. Since even basal levels of growth were not achieved in the presence of these compounds, it must be concluded that they are inhabitory to Isolate #1. The mechanisms by which these five classes of compounds inhibited H_2S production, and therefore growth, have not been further investigated.

It can be concluded from this study that Isolate #1 makes better use of simple reduced compounds as electron donors, than it does preformed amino acids, TCA-intermediates, or sugars. This is good evidence that in nature, the organism could grow by scavenging simple metabolic end-products from aerobic and fermenting organisms.

H. Attempts to Extract Natural Alkanes from Isolate #1

Alkanes have been reported in extracts of algae and from bacteria such as Vibrio ponticus growing on a lactate + salts medium (Meinshien, 1969). Since, as mentioned in the Literature Review, sulfate-reducers have been implicated as active organisms in the process of petroleum genesis, an attempt was made to determine whether Isolate #1 accumulated alkane material during normal metabolism. Cells were grown on both Medium #3 and Medium #4 for 72 hours at 30°, after which they were harvested and analyzed for the presence of alkane or naphthenic materials. As none were found, it is likely that under these conditions, this isolate does not synthesize significant amounts of hydrocarbon.

I. Fatty Acid Composition of the Lipid Component of Isolate #1

A review of the literature on the physiology and composition of a generalized Desulfovibrio cell reveals that there is a lack of information concerning the fatty acid composition of such a cell. This knowledge could be of use in understanding the metabolism of this important group of microorganisms and could eventually aid in their numerical taxonomy.

A study of the effect of age and growth medium on the fatty acid composition of cells of Isolate #1 was undertaken using batch culture. Cultures were grown on Medium #3 (yeast extract 1.0 g/l, sodium lactate 4.5 ml/l, Na_2SO_4 4.0 g/l) and on Medium #4 (yeast extract 3 g/l, sodium lactate 1.5 ml/l, Na_2SO_4 4g/l). Cells were harvested by centrifugation after 1,2,4, and 7 days growth, and analyzed for protein and fatty acid concentration.

The cell protein concentrations obtained in both media, as a function of time, are presented in Table III. Although the growth rate appeared to be slower on Medium #3 than on Medium #4, a slightly higher cell yield was obtained on the latter medium.

The concentrations of identified fatty acids extracted from cells of Isolate #1 grown on Medium #3 are shown in Table IV. The most abundant fatty acids present in the cellular lipid components after 7 days growth were hexadecanoic and octadecanoic acids. The concentration of tetradecanoic and the unsaturated cis-9-hexadecenoic and cis-9-octadecenoic acids comprised less than one-fifth of the total identified fatty acids. After 7 days growth the 5 known fatty acids were present in excess of 1% by weight of the protein content of the cells. If one assumes that the protein constitutes approximately 50% of the cell weight, then these fatty acids made up slightly more than 0.5% of the total cell weight.

Table V lists 7 unidentified components, as a function of GLC retention time, from cells of Isolate #1 grown on Medium #3. These components were methylated and resolved on an EGS column. The retention times of known fatty acids have been included for comparative purposes.

Table VI shows the quantitative analysis of 5 known fatty acids extracted from the cell pellets of the culture grown on Medium #4. Again, as was the case with growth on Medium #3, hexadecanoic and octadecanoic acids were the most abundant fatty acids extracted. However, in this case, Medium #4 (containing a higher yeast extract concentration) stimulated a proportionally higher production of

TABLE III. CELL PROTEIN CONCENTRATIONS DURING GROWTH ON MEDIUM #3 AND #4

	Micrograms Cell Protein/ml	
Incubation	Medium #3	Medium #4
0	6.2	6.2
1	58.5	47.0
2	57.0	63.5
4	57.1	67.0
7	42.0	65.0

TABLE IV. CONCENTRATIONS OF FIVE IDENTIFIED FATTY ACIDS EXTRACTED FROM CULTURES OF ISOLATE #1 GROWN ON MEDIUM #3 AS A FUNCTION OF CULTURE AGE

	ng of fatty acid/μg of cell protein			
Fatty Acid	Day 1	Day 2	Day 4	Day 7
Tetradecanoic acid (C-14)	0.24	0.24	0.32	0.84
Hexadecanoic acid (C-16)	2.16	2.44	3.08	4.91
CIS-9-hexadecenoic acid (C-16:1)	0.40	0.60	0.44	0.40
Octadecanoic acid (C-18)	2.55	2.92	3.80	5.01
CIS-9-octadecenoic acid (C-18:1)	0.88	0.34	0.90	0.80
Total ng fatty acid/μg protein	6.23 (0.62)	6.54 (0.65)	8.54 (0.85)	11.96 (1.19)

Note: Values in brackets are the total concentrations of the five identified fatty acids expressed as a weight percentage of the cellular protein.

Cultures of Isolate #1 were grown in one liter storage bottles on Media #3 and #4. On days 1, 2, 4, and 7, 200 ml volumes of cells were harvested by centrifugation, washed 4 times in 3 mM phosphate buffer (pH 6.5) and finally suspended in 0.1 N HCl. These preparations were extracted, the extracts methylated, and analyzed as described in Materials and Methods.

TABLE V. UNIDENTIFIED LIPID COMPONENTS EXTRACTED FROM ISOLATE #1 GROWN ON MEDIUM #3 AS A FUNCTION OF CULTURE AGE

	Relative Percentage[2]			
Retention Time (min)	Day 1	Day 2	Day 4	Day 7
6.20 (C_{14})[1]	-	-	-	-
7.61	38.8	36.7	40.5	100.
8.20 (C_{15})[1]	-	-	-	-
11.64 ($C_{16:o}$)[1]	-	-	-	-
13.17 ($C_{16:1}$)[1]	-	-	-	-
14.46	45.3	48.6	42.6	100.
15.71	100.	47.8	74.9	72.5
17.47	100.	15.9	30.4	76.3
19.07	0.0	100.	0.0	0.0
22.36 ($C_{18:0}$)[1]	-	-	-	-
24.50 ($C_{18:1}$)[1]	-	-	-	-
27.60	0.0	100.	0.0	0.0
40.14	0.0	100.	0.0	0.0

Total number of unknown components: 7

1. The retention times of the known fatty acids are included for reference points.

2. The percentage values given in the table result from giving the highest integrated value of each component the ranking of 100%. All other values of the respective components are then expressed as a percentage of the highest ranking value.

TABLE VI. CONCENTRATIONS OF FIVE IDENTIFIED FATTY ACIDS EXTRACTED FROM CULTURES OF ISOLATE #1 GROWN ON MEDIUM #4 AS A FUNCTION OF CULTURE AGE

	ng of fatty acid/µg of cell protein			
Fatty Acid	Day 1	Day 2	Day 4	Day 7
Tetradecanoic acid (C-14)	0.0	0.90	0.60	0.40
Hexadecanoic acid (C-16)	1.50	4.80	9.40	6.90
CIS-9-hexadecenoic acid (C-16:1)	0.51	1.60	3.40	0.90
Octadecanoic acid (C-18)	0.40	1.70	3.60	3.10
CIS-9-octadecenoic acid (C-18:1)	0.20	0.70	2.00	2.20
Total ng fatty acid/µg protein	2.60 (0.26)	9.70 (0.97)	19.0 (1.90)	13.5 (1.35)

NOTE: Values in brackets are the total concentrations of the five identified fatty acids expressed as a weight percentage of the cellular protein.

hexadecanoic acid. This was not due to significant absorption of fatty acids from the growth medium, since control extractions of both Medium #3 and Medium #4 yielded very low concentrations of fatty acids.

Table VII lists 5 unidentified components extracted from the culture grown on Medium #4 as a function of culture age. In this case, the unknown components were fewer in number, but all achieved their maximum concentration after 4 days of culture incubation.

Since, as mentioned previously, extraction of both growth media revealed very little pre-formed lipid being available to the growing cultures, most of the identified and unidentified lipids were synthesized *de novo* by cells of Isolate #1. It is also apparent, that the growth medium and length of incubation period strongly influenced the variety and amounts of lipid components synthesized. The reasons why yeast extract stimulated higher production rates of fatty acids was not investigated.

II. Effects of Levels of Electron Donor and Electron Acceptor on the Growth and Metabolism of Isolate #1 Grown in Continuous Culture at a Constant Dilution Rate

Continuous culture techniques were used in order to obtain data from exponentially-growing cells. The study of metabolism using cells produced by batch culture techniques is limited by the fact that the environment, and thus growth rate, is continually changing. The use of the Dawson-Type of chemostat, since it relies on a manometric system to control liquid levels and requires continuous sparging with a gas, resulted in a continuous removal of H_2S. This prevented the development of toxic levels of this product of metabolism, which is a major problem in the growth of these bacteria under batch culture techniques.

TABLE VII. UNIDENTIFIED LIPID COMPONENTS EXTRACTED FROM ISOLATE #1 GROWN ON MEDIUM #4 AS A FUNCTION OF CULTURE AGE

	Relative Percentage[2]			
Retention Time (min)	Day 1	Day 2	Day 4	Day 7
6.20 (C_{14})[1]	-	-	-	-
7.61	10.6	52.3	100.	91.1
8.20 (C_{15})[1]	-	-	-	-
10.30	0.0	26.2	100.	40.0
11.64 ($C_{16:0}$)[1]	-	-	-	-
13.17 ($C_{16:1}$)[1]	-	-	-	-
14.46	17.8	65.8	100.	87.5
15.71	18.5	51.0	100.	27.5
17.47	15.5	47.9	100.	97.8

Total number of unknown components: 5

1. The retention times of the known fatty acids are included for reference points.

2. The percentage values given in the table result from giving the highest integrated value of each component the ranking of 100%. All other values of the respective components are then expressed as a percentage of the highest ranking value.

The data obtained from chemostat studies more accurately reflected the role of lactate, sulfate, and yeast extract in the metabolism of this isolate.

The cellular responses of Isolate #1 grown on a series of media (Media No. 1 to 5) of varying sulfate, lactate, and yeast extract concentrations were compared in these experiments. The growth rate was maintained at 0.66 hr^{-1} and culture pH values remained at 8.2-8.4 throughout the experimental series.

A. Consumption of Available Lactate and Sulfate by Isolate #1 as a Function of Influent Medium

Table VIII summarizes the influent and effluent concentrations of lactate and sulfate as a function of medium composition. It is evident that in three (No. 1, 4, 5) of the five media tested, the primary electron donor (lactate) was growth-limiting; whereas the consumption rates for sulfate never exceeded 57% of that present. The tripling of the lactate concentration in Medium No. 2 and 3 resulted in significant residual levels of lactate and sulfate; although increased levels of utilization were noted. Further utilization of lactate was not stimulated by doubling the sulfate concentration (Medium #3); therefore, neither lactate nor sulfate were limiting growth in Media No. 2 or 3. The role of yeast extract in the medium and its influence on dissimilatory sulfate reduction and lactate utilization is indicated by comparing the results obtained with Media No. 1, 4, and 5. Full utilization of high concentrations of lactate only occurred where increased levels of yeast extract were present. Thus, although lactate appeared most often to be the growth-limiting nutrient, a certain ratio of yeast extract to lactate is required for maximum lactate utilization.

TABLE VIII. CONSUMPTION OF LACTATE AND SULFATE AS A FUNCTION OF INFLUENT MEDIUM COMPOSITION

	Ratio[1]				
Medium	Lactate:	Yeast Extract:	Sulfate	% Lactate Used	% Sulfate Used
1	1	1	1	99.4	38.5
2	3	1	1	57.0	52.7
3	3	1	2	52.9	31.8
4	1	3	2	98.3	21.5
5	3	3	2	98.6	56.9

[1] Ratios refer to the concentrations of lactate, yeast extract, and sulfate used, relative to those concentrations used in Medium #1, i.e., lactate (60% syrup): 1.5 ml/l

yeast extract: 1 g/l

Na_2SO_4 : 2 g/l

These results suggest that yeast extract concentrations were limiting growth in Media #'s 2 and 3.

B. Production of Cellular Constituents

The RNA, DNA and protein contents of the cells produced on the five media are presented in Table IX. Since protein concentrations never exceeded 134 µg/ml, it is obvious that cell concentrations were never very high as compared with other cell systems grown in continuous culture under aerobic conditions (e.g., Salmonella typhimurium: 0.5 - 1.1 mg dry weight of cells/ml of continuous culture; Kjeldgaard, 1967). However, the five media used did support differing levels of cell production.

The results for RNA, DNA, and protein are best compared with the basal levels achieved using Medium #1. The use of Medium #2, containing three times the lactate concentration, did not cause a doubling of protein production but did roughly double the levels of RNA and DNA. Medium #3, containing enhanced sulfate levels and lactate equivalent to Medium #2, did not stimulate higher levels of RNA, DNA, or protein production, indicating that sulfate certainly was not limiting growth in Medium #2. This would again suggest that a maximal response was not achieved due to yeast factor(s) becoming limiting. The use of Medium #4 clearly demonstrated that tripling yeast extract, while leaving lactate at basal levels, did not give nearly the response seen when lactate levels were tripled. This indicates that while yeast extract can cause increases in the efficiency of utilization of the lactate present (resulting in 69 µg of cellular protein/ml as opposed to 54 µg/ml from Medium #1), it cannot replace lactate as a primary

TABLE IX. PRODUCTION OF CELLULAR CONSTITUENTS AS A FUNCTION OF THE COMPOSITION OF THE INFLUENT MEDIUM

Medium	Ratio[1] Lactate	Yeast Extract	Sulfate	μg/ml Protein	RNA[2]	DNA[3]
1	1	1	1	54.0	5.90	3.60
2	3	1	1	81.0	11.8	7.00
3	3	1	2	80.8	12.3	8.00
4	1	3	2	69.2	8.60	5.10
5	3	3	2	134.0	17.1	9.00

[1] Ratios refer to the concentrations of lactate, yeast extract, and sulfate used relative to those concentrations used in Medium #1, i.e. lactate (60% syrup): 1.5 ml/m

Yeast extract: 1 g/l

Na_2SO_4: 2 g/l

[2] Estimate of μg RNA/ml obtained by multiplying μg Ribose x 2.28.

[3] Estimate of μg DNA/ml obtained by multiplying μg Deoxyribose x 2.44.

source of carbon. This result again argues strongly for the role of lactate in this system as being the primary source of electrons and carbon. The triple lactate and yeast extract in Medium #5, as compared to the basal Medium #1, did not quite cause a tripling of either protein or DNA levels, but did allow RNA levels to triple. This effect also applied to total sulfate and lactate consumption (Table VIII). These results suggest that substrate concentrations (i.e., lactate + yeast extract) are the growth limiting factors in this media design.

Table X lists the cellular RNA:DNA ratios measured during the growth of Isolate #1 on the five media. These ratios, with one exception, remained within the range of 1.5-1.6. Although it was evident that control of nucleic acid synthesis and metabolism did exist under these circumstances, the RNA:DNA ratios indicate that the isolate contained DNA in amounts which were inordinantly high when compared to levels of RNA. Normal RNA:DNA ratios in E. coli lie in the region of 10:1 (Kjeldgaard, 1967). This means that RNA turnover rates are very rapid in Isolate #1, or that cells retain large amounts of genome under most given growth situations. No attempts were made to determine the average number of genomes present in these cells. The protein:RNA and protein:DNA ratios did not exhibit any significant trends as a function of the growth medium used by the organism.

Viable counts obtained during growth of Isolate #1 on the five media are listed in Table XI. These results, when compared with data shown in Table IX, show some disturbing inconsistencies. For instance, in shifting from Medium #1 to #2, cellular protein concentrations increased some 50% while viable counts/ml showed no change. Use of

TABLE X. PROTEIN:NUCLEIC ACID RATIOS AS A FUNCTION OF THE COMPOSITION OF THE INFLUENT MEDIUM

	Ratio[1]			Protein	Protein	RNA
Medium	Lactate:	Yeast Extract:	Sulfate	DNA	RNA	DNA
1	1	1	1	15.0	9.2	1.6
2	3	1	1	11.5	6.8	1.6
3	3	1	2	10.1	6.5	1.5
4	1	3	2	13.5	8.0	1.6
5	3	3	2	14.8	7.8	1.9

[1] Ratios refer to the concentrations of lactate, yeast extract, and sulfate used, relative to those concentrations used in Medium #1, i.e., lactate (60% syrup): 1.5 ml/l

yeast extract: 1 g/l

Na_2SO_4: 2 g/l

TABLE XI. VIABLE COUNT AS A FUNCTION OF THE COMPOSITION OF THE INFLUENT GROWTH MEDIUM

	Ratio[1]			Viable Count	Femtograms/cell		
Medium	Lactate:	Yeast Extract:	Sulfate	/ml of Effluent	Protein	RNA	DNA
1	1	1	1	2.8×10^8	192	21.0	12.8
2	3	1	1	2.8×10^8	289	42.0	36.0
3	3	1	2	4.0×10^8	202	30.7	20.0
4	1	3	2	2.8×10^8	246	30.7	18.2
5	3	3	2	1.1×10^9	121	15.5	8.18

---

[1] Ratios refer to the concentrations of lactate, yeast extract, and sulfate used, relative to those concentrations used in Medium No. 1, i.e., lactate (60% syrup): 1.5 ml/l
yeast extract: 1 g/l
Na_2SO_4: 2 g/l

NOTE: All black colonies were counted after 4 days incubation at 30°.

Medium #3 did not cause any increase in cellular protein concentration, but did yield an apparent 42% increase in viable counts. Medium #4 caused viable counts to fall apparently to the basal level achieved using Medium #1 while the protein concentration remained 27% above basal levels. Finally, use of Medium #5, compared to Medium #1, yielded a 150% increase in protein production and an apparent 300% increase in viable counts. This, for instance, should lead to a net decrease in cell size; yet a cursory examination of Table XII and Figure 4 indicates from cell measurements that Medium #3 actually produced slightly larger cells when compared with Media No. 1, 3, and 4. This is taken as an indication that viable count studies with this organism reveal only gross population changes; such studies are prone to errors from cells adhering and manifesting themselves as single colonies. Figure 4 also shows a great similarity in cell size distribution within populations grown on Media No. 1, 3, and 4. To some extent, the sizing of cells by phase contrast microscopy may also lead to error, especially in the case of measuring cells over 3 μm in length, where the length is actually made up of two or more cells adhering end-to-end. However, E.M. studies (Plate 2(a)B) revealed examples of long cells showing three or more half-cycles and no evidence of cross wall formation, indicating that they were in fact single viable units.

These results demonstrate that growth of this Desulfovibrio isolate is best followed by assaying cellular constituents rather than carrying out viable count studies.

C. Growth Yield Calculations

The literature concerning molar growth yields of Desulfovibrio sp. was reviewed in the Literature Survey, as were the problems inherent

TABLE XII. SUMMARY OF CELLULAR LENGTH (μm) AND HALF-CYCLE DISTRIBUTION AS A FUNCTION OF THE COMPOSITION OF THE INFLUENT GROWTH MEDIUM

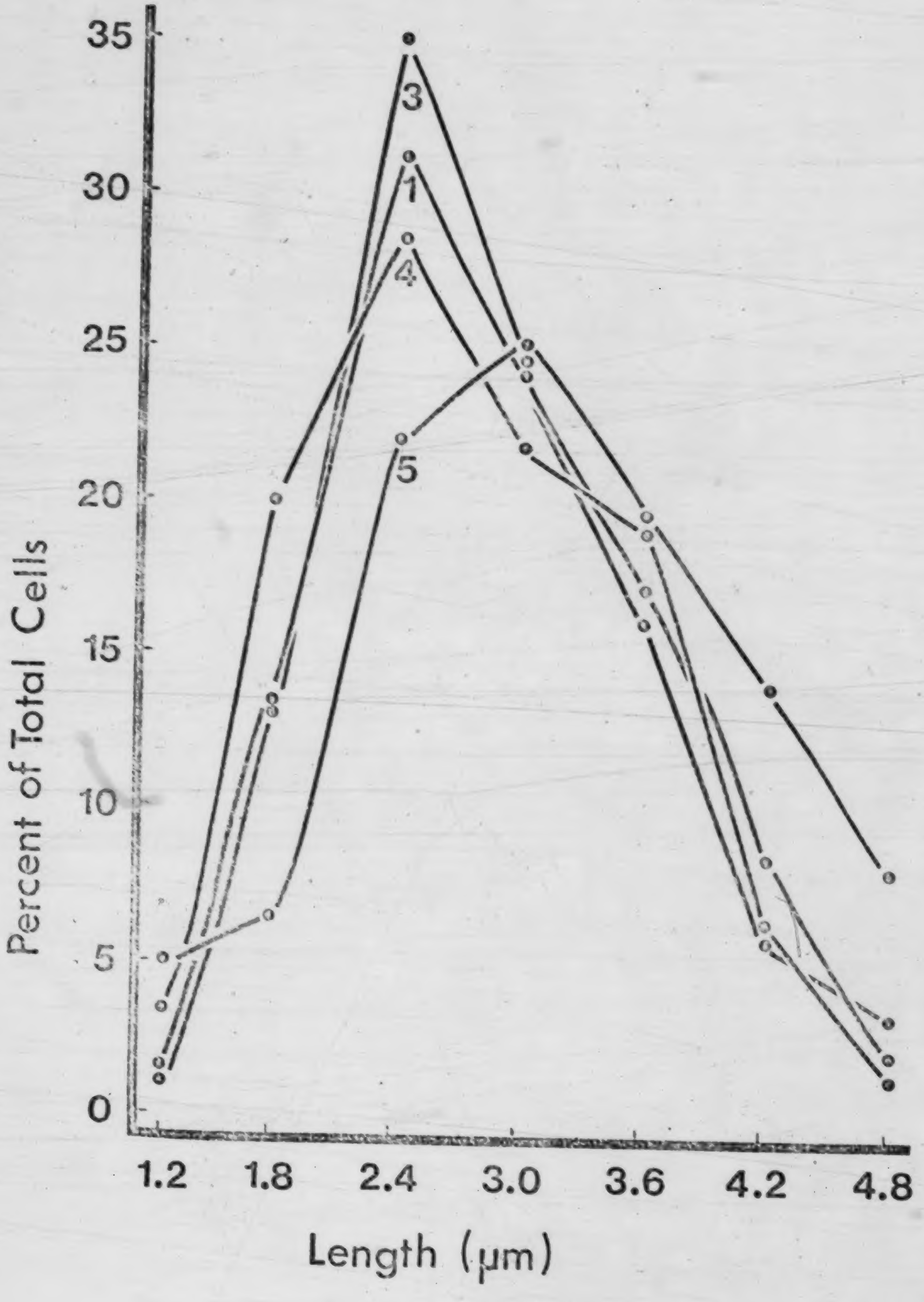
		Percent of Total			
Length	Number of 1/2 cycles	Medium #1	Medium #3	Medium #4	Medium #5
1.2 μm	1	1.6	1.1	3.4	4.9
		Σ1.6	Σ1.1	Σ3.4	Σ4.9
1.8 μm	1	11.6	12.3	15.4	4.9
	2	1.6	1.1	4.4	1.5
		Σ13.2	Σ13.4	Σ19.8	Σ6.5
2.4 μm	1	12.1	9.5	6.9	8.8
	2	19.3	24.0	21.8	12.1
	3	0.0	1.6	0.0	1.1
		Σ31.4	Σ35.1	Σ28.7	Σ22.0
3.0 μm	1	2.2	0.0	0.5	1.1
	2	16.5	18.5	20.8	18.7
	3	5.5	6.1	0.5	5.5
		Σ24.2	Σ24.6	Σ21.8	Σ25.3
3.6 μm	2	8.8	6.1	12.9	5.5
	3	8.2	10.1	6.0	13.8
		Σ17.0	Σ16.2	Σ18.9	Σ19.3
4.2 μm	2	1.6	1.1	2.0	1.1
	3	6.0	4.4	4.0	12.1
	4	0.5	0.0	0.0	0.5
		Σ8.1	Σ5.5	Σ6.0	Σ13.7
4.8 μm	3	1.6	1.6	1.0	2.7
	4	0.0	1.6	0.0	4.9
		Σ1.6	Σ3.2	Σ1.0	Σ7.6

Figure 4

SIZE DISTRIBUTION OF VIABLE CELLS
AS A FUNCTION OF GROWTH MEDIUM

All plotted points are the summed values for each measured length obtained from Table XII.

1 = Cells grown on Medium #1
3 = Cells grown on Medium #3
4 = Cells grown on Medium #4
5 = Cells grown on Medium #5


35
30
25
20
15
10
5
0
Percent of Total Cells
1.2
1.8
2.4
3.0
3.6
4.2
4.8
Length (µm)
3
1
4
5

in such calculations. Some of the data obtained in this study were re-calculated to give yield values, and are presented in Table XIII. The obvious conclusion reached as a result of such calculations is that a rough stoichiometry of 2:1 should exist between molar quantitites of lactate and sulfate utilized. For the most part, the data shown in Table XIII agree with this conclusion, although use of excess lactate versus basal sulfate and yeast extract (Media No. 2 and 3) gave evidence of primary electron donor wastage (ratios of 2.5:1 and 2.2:1 respectively). Another interesting observation can be drawn from the column for $Y_{(lactate)}$ values. If one accepts the statement of Senez (1962) that Desulfovibrio sp. generate 1 mole of ATP (net)/mole of lactate utilized, this column could be re-titled $Y_{(ATP)}$ with respect to protein. If one also assumes that these cells maintain about 50% by weight of cellular protein, doubling of the values shown should give hypothetical $Y_{(ATP)}$ values with respect to dry cell weight. These values vary from 8.0 to 11.8. Senez quotes a value of approximately 10 for the Canet strain of Desulfovibrio. Based on the rough calculations from data in Table XIII, it would seem that such a $Y_{(ATP)}$ value might be reasonable under these growth conditions.

D. Production of Reduced Sulfur Intermediates as a Function of the Composition of the Influent Growth Medium

The data in Table XIV show the amounts of sulfide (solution and sparged), thiosulfate, and sulfite found in effluents produced during growth studies on the five media. As was expected, sulfide was the major product of sulfate reduction. It is also apparent that from 40% to 50% of the sulfide remained in solution even with continuous sparging of nitrogen gas. The reason for this is that at a pH of

TABLE XIII. PROTEIN YIELDS AS A FUNCTION OF THE COMPOSITION OF THE INFLUENT MEDIUM

	Ratio[1]			Cellular Protein Yield[2]		Ratio $\frac{\mu\text{Moles Lactate Utilized}}{\mu\text{Moles Sulfate Utilized}}$
Medium	Lactate:	Yeast Extract:	Sulfate	$Y_{(Sulfate)}$	$Y_{(Lactate)}$	
1	1	1	1	10.0	5.0	1.9
2	3	1	1	10.4	4.2	2.5
3	3	1	2	8.8	4.0	2.2
4	1	3	2	10.9	5.9	1.8
5	3	3	2	7.7	4.0	1.9

[1] Ratios refer to the concentrations of lactate, yeast extract, and sulfate used, relative to those concentrations used in Medium No. 1, i.e., lactate (60% syrup): 1.5 ml/1

yeast extract: 1 g/1

Na_2SO_4: 2 g/1

[2] All yield values expressed as grams of cellular protein/mole of sulfate or lactate utilized.

TABLE XIV. PRODUCTION LEVELS OF EFFLUENT SULFIDE, SULFITE, THIOSULFATE AND SPARGED H_2S AS A FUNCTION OF THE COMPOSITION OF THE INFLUENT MEDIUM

	Ratio[1]			Sulfide in Effluent		Sparged H_2S[2]		Sulfite in Effluent		Thiosulfite in Effluent	
Medium	Lactate:	Yeast Extract:	Sulfate	μmoles/hr	%/hr[3]	μmoles/hr	%/hr[3]	μmoles/hr	%/hr[3]	μmoles/hr	%/hr[3]
1	1	1	1	275	12.9	252	11.8	0.0	0.0	0.0	0.0
2	3	1	1	267	12.5	366	17.1	5.70	0.27	33.1	3.10
3	3	1	2	316	7.68	446	10.8	21.6	0.52	46.0	2.21
4	3	3	2	907	20.8	836	19.2	73.4	1.68	126.0	5.78

[1] Ratios refer to the concentrations of lactate, yeast extract, and sulfate used, relative to those concentrations used in Medium #1, i.e., lactate (60% syrup): 1.5 ml/l

yeast extract: 1 g/l

Na_2SO_4: 2 g/l

[2] $$\text{Sparged } H_2S \text{ \%/hr} = \frac{\mu\text{moles } H_2S\text{/hr}}{\mu\text{moles total influent sulfur/hr}} \times 100$$

[3] $$\text{\%/hr} = \frac{\mu\text{moles of species sulfur/hr}}{\mu\text{moles total influent sulfur/hr}} \times 100$$

In the case of thiosulfate, final value is multiplied by 2.0 since thiosulfate contains two sulfur atoms.

8.2-8.4, only about 3% of the sulfide is present as H_2S (see Figure 5). Thus, even with constant sparging, the reaction rate of the conversion of ionized sulfide to H_2S is too slow to free the system of sulfide. This is an important factor in maintaining a reduced environment in the chemostat while minimizing the concentration of this toxic metabolite. Maximum total production of sulfide occurred during the use of Medium #5.

Thiosulfate ion was the most abundant intermediate of sulfur found in solution during growth on Media No. 2, 3, 4, and 5. That thiosulfate should accumulate is not unexpected, since it apparently is the last intermediate in the dissimilatory route. Moreover, it should be more abundant than sulfite since sulfite is the product of an endergonic activation-reduction which would not occur unless sulfite was in fact needed as a subsequent electron acceptor. As well, sulfite is apparently constantly re-cycled within the dissimilatory route, again indicating that it is a transitory intermediate in the reduction pathway.

No thiosulfate or sulfite could be detected in effluents of Medium #1. This most likely was due to the fact that they were in such low concentrations that they would not have been detected by the iodimetry procedures employed. Table XV shows the recoveries of the total influent sulfate-sulfur along with the total per hour production of sulfide end-product and sulfur intermediates. Between 80 and 100 percent of the total influent sulfur could be accounted for, depending on which of the five growth media were being used. The lowest recovery of sulfur occurred during the use of Medium #2 (rich in electron donor

Figure 5

FORMS OF SOLUBLE SULFIDES IN AQUEOUS SOLUTION AS A FUNCTION OF pH

Equations for derivation of curves:

$$\frac{[H_2S_{aq.}]}{[T.S.S.]} = \frac{1}{1 + \frac{K_1}{[H^+]} + \frac{K_1K_2}{[H^+]^2}}$$

$$\frac{[HS^-]}{[T.S.S.]} = \frac{1}{1 + \frac{[H^+]^2}{K_1} + \frac{K_2}{[H^+]}}$$

$$\frac{[S^=]}{[T.S.S.]} = \frac{1}{1 + \frac{[H^+]^2}{K_1K_2} + \frac{[H^+]}{K_2}}$$

T.S.S. = total soluble sulfide

$H_2S \xrightarrow{K_1} HS^- + H^+$ ($K_2 = 9.1 \times 10^{-8}$; pK 7.04)

$HS^- \xrightarrow{K_2} S^= + H^+$ ($K_2 = 11.96 \times 10^{-12}$; pK 11.96)

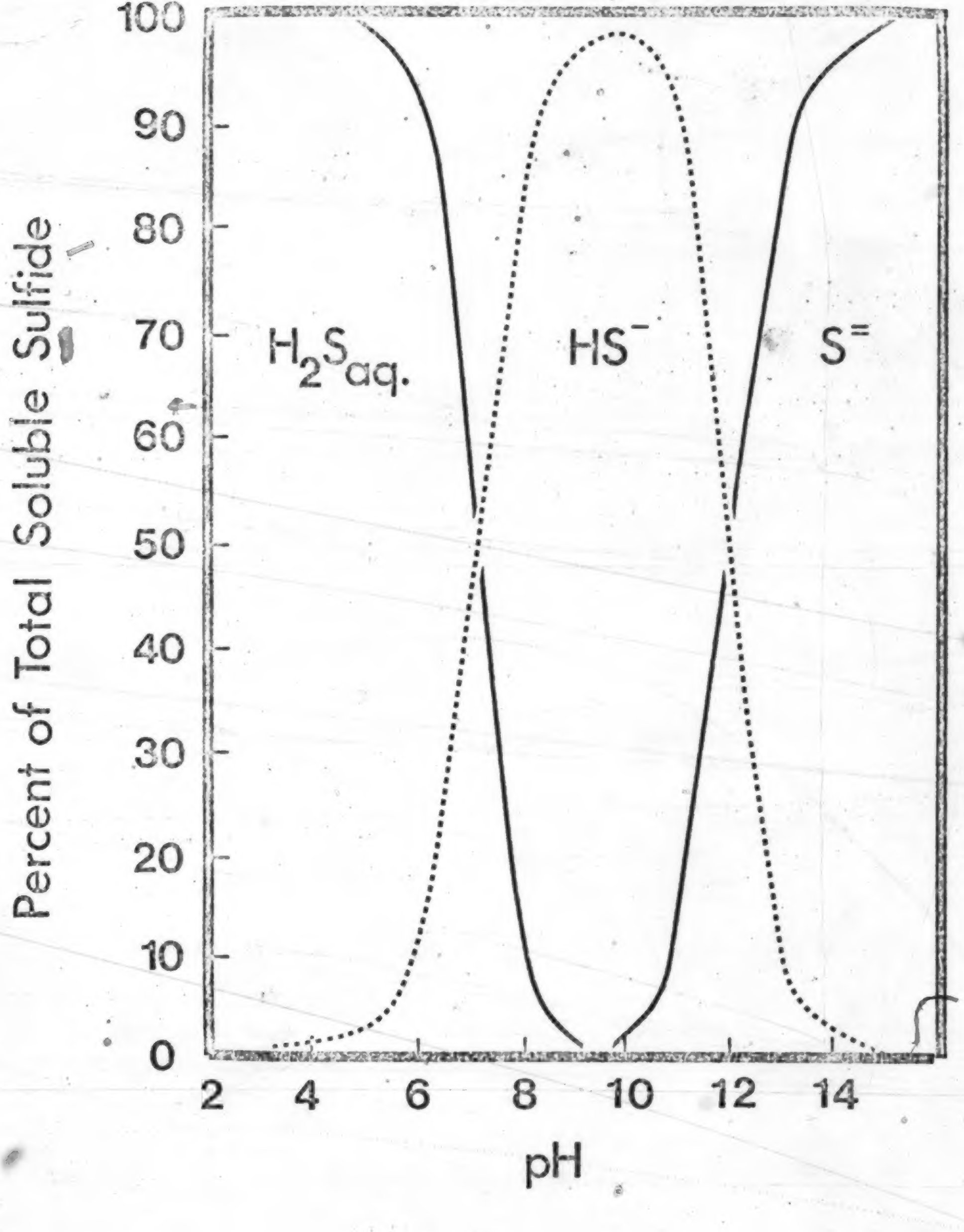

Percent of Total Soluble Sulfide
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
pH
$H_2S_{aq.}$
HS^-
$S^=$

TABLE XV. SULFUR RECOVERIES DURING GROWTH ON THE FIVE MEDIA

	Ratio[1]			Influent[2]	Effluent[2]		Gas[2]	Recovery[2]	
Medium	Lactate:	Yeast Extract:	Sulfate	Sulfate - S/hr	Reduced S/hr	Unused Sulfate - S/hr	H_2S Sparged/hr	Total/hr	% of Influent
1	1	1	1	2131	275	1310	252	1837	86.3
2	3	1	1	2130	399	1012	366	1717	80.7
3	3	1	2	4142	430	2808	446	3684	89.4
4	1	3	2	4202	475	3297	431	4203	100.0
5	3	3	2	4348	1218	1872	836	3926	90.3

[1] Ratios refer to the concentrations of lactate, yeast extract, and sulfate used, relative to those concentrations used in Medium #1, i.e., lactate (60% syrup): 1.5 ml/l

Yeast extract: 1 g/l

Na_2SO_4: 2 g/l

[2] All values expressed in µmoles.

with only basal levels of sulfate and yeast extract). Sulfur was completely accounted for when Medium #4 (rich in yeast extract and deficient in lactate) was employed in the chemostat. It is highly unlikely that the assays for sulfate and reduced intermediates were giving inconsistent results, since it was possible to recover all of the intermediates when artificially added to culture media. It is more likely that under certain conditions (e.g., the use of Medium #2) either a very truncated route of dissimilatory sulfate reduction was occurring, giving more intermediates of sulfur than have classically been reported, or that significant levels of trithionate were accumulating. On the basis of ATP conservation, one would tend to favor the pushing of dissimilatory sulfate reduction to completion, since this would allow the maximum acceptance of electrons with the minimum requirements for activation of sulfate which costs the equivalent of 2 ATPs/sulfate molecule. On this basis, the formation of many reduced sulfur intermediates would be energetically wasteful.

Table XV reveals that all sulfur was accounted for during the use of Medium #4 which was deficient in lactate but abundant in yeast extract. It is tempting to speculate that the presence of factor(s) from yeast extract pushed the process of dissimilatory sulfate reduction toward completion. Thus, most of the sulfur would appear as either sulfide, sulfite, thiosulfate, or unused sulfate. The amount of trithionate present would in all likelihood, be sufficiently small to be within the experimental error range of the other determinations. Therefore, the data from Table XV suggest, but do not necessarily prove, that factor(s) from yeast extract help to drive sulfate reduction to completion. This could also be caused indirectly by the factor(s) from the yeast extract stimulating overall cell growth, therefore increasing the energy requirements of the cells.

E. Detection of ^{34}S Enrichment in Residual Sulfate as a Function of Influent Growth Medium Composition

A summary of the $\delta^{34}S$ values of the influent and effluent sulfate-sulfur from the various media used are presented in Table XVI. It is clear from the table that only three media stimulated any significant levels of fractionation as reflected by enrichment of ^{34}S in the effluent sulfate-sulfur. In all three cases, sulfate was present at twice the basal concentration. In addition, growth on Media No. 2 or 3 produced about the same amount of sparged H_2S and the effluent sulfate from these two runs gave almost identical $\delta^{34}S$ values. Growth under conditions of Medium #5 gave approximately double the amount of sparged H_2S (in comparison with the previous two media) and in fact, the effluent sulfate-sulfur experienced a doubling of its $\delta^{34}S$ value. Thus, it would seem that the degree of fractionation varied directly with the amount of dissimilatory sulfate reduction. In the case of growing on single strength Butlins or on Medium #2, no significant fractionation occurred, judging from the $\delta^{34}S$ value of the effluent sulfate-sulfur. Both of these growing conditions were not deficient in sulfate since the effluent sulfate concentrations were significant in both cases. At the dilution rate (0.06 hr^{-1}) used throughout this series of experiments, it would seem that only under conditions of increased lactate, sulfate, and yeast extract does any reasonable level of sulfur fractionation occur. The overall results suggest that all three substrates are important with respect to fractionation, and that the sole dependence of fractionation on large starting concentrations

TABLE XVI. $\delta^{34}S$ VALUES OF INFLUENT AND EFFLUENT SULFATE-SULFUR AS A FUNCTION OF THE GROWTH MEDIUM COMPOSITION

	Ratio[1]			$\delta^{34}S$ Influent	$\delta^{34}S$ Effluent
Medium	Lactate:	Yeast Extract:	Sulfate	Sulfate-Sulfur	Sulfate-Sulfur
1	1	1	1	-0.03	+0.06
2	3	1	1	+0.06	-0.04
3	3	1	2	+0.05	+1.90
4	1	3	2	-0.03	+2.00
5	3	3	2	+0.10	+4.10

[1] Ratios refer to the concentrations of lactate, yeast extract, and sulfate used, relative to those concentrations used in Medium #1, e.g., lactate (60% syrup): 1.5 ml/l

yeast extract: 1 g/l

Na_2SO_4: 2 g/l

of sulfate would not seem to be the case in this study.

Although sparged H_2S was trapped as ZnS and samples were subjected to mass spectrometric analyses, the results were not included in Table XVI. This is due to the fact that in the time between trapping and eventual analysis, some auto-oxidation to other sulfur species had occurred. Although re-precipitation of the sulfate as $BaSO_4$ was carried out, other species were still probably present. The analyses of the ZnS + $BaSO_4$ precipitates gave $\delta^{34}S$ values all within the range of (-)9.1 - (-)10.0. This was much more fractionation than was seen in the effluent sulfate-sulfur, which would indicate that some chemical fractionation had occurred in the zinc acetate trapping solution. Thus any data concerning ^{34}S values in sulfide would be rendered useless.

III. Effect of Dilution Rate on the Growth of Isolate #1 under Continuous Culture Conditions

The data presented in this section were obtained by varying the dilution rate and maintaining the composition of the influent medium at a constant level of lactate, yeast extract, and sulfate. Medium #6, containing lactate, yeast extract, and sulfate in a ratio of 3:2:1, as compared to basal Medium #1, was designed to maintain the primary electron donor (lactate) and acceptor (sulfate) at levels which would not become limiting at the various dilution rates studied. The yeast extract level, based on the initial chemostat study, was chosen to give an intermediate level of growth. Single strength sulfate was used, since the previous study had shown that this was sufficient to have excess sulfate remaining in the effluent.

A. Growth Conditions

The influent flow rate to the chemostat was varied using a peristaltic pump whose flow rate characteristics are shown in Figure 6. This chemostat was thus able to provide a dilution rate range of 0.012 hr^{-1} to 0.316 hr^{-1}, corresponding to mean culture residence times of 41 to 1.58 hours.

The pH values of chemostat effluents are presented in Figure 7. The values varied from pH 8.8. at a dilution rate of 0.012 hr^{-1} to pH 7.3 at the highest dilution rate of 0.316 hr^{-1}. The steady decrease in pH was a result of less H_2S being produced /ml of growth medium at each of the increasing dilution rates, even though the overall hourly H_2S production did increase for three of the measured dilution rates (Figure 5). In dissimilatory sulfate-reducer growth, the acidic reaction resulting from the production of organic acids is neutralized

Figure 6

FLOW RATE CHARACTERISTICS OF THE CHEMAPAC PERISTALIC PUMP USED IN THE DILUTION RATE STUDY

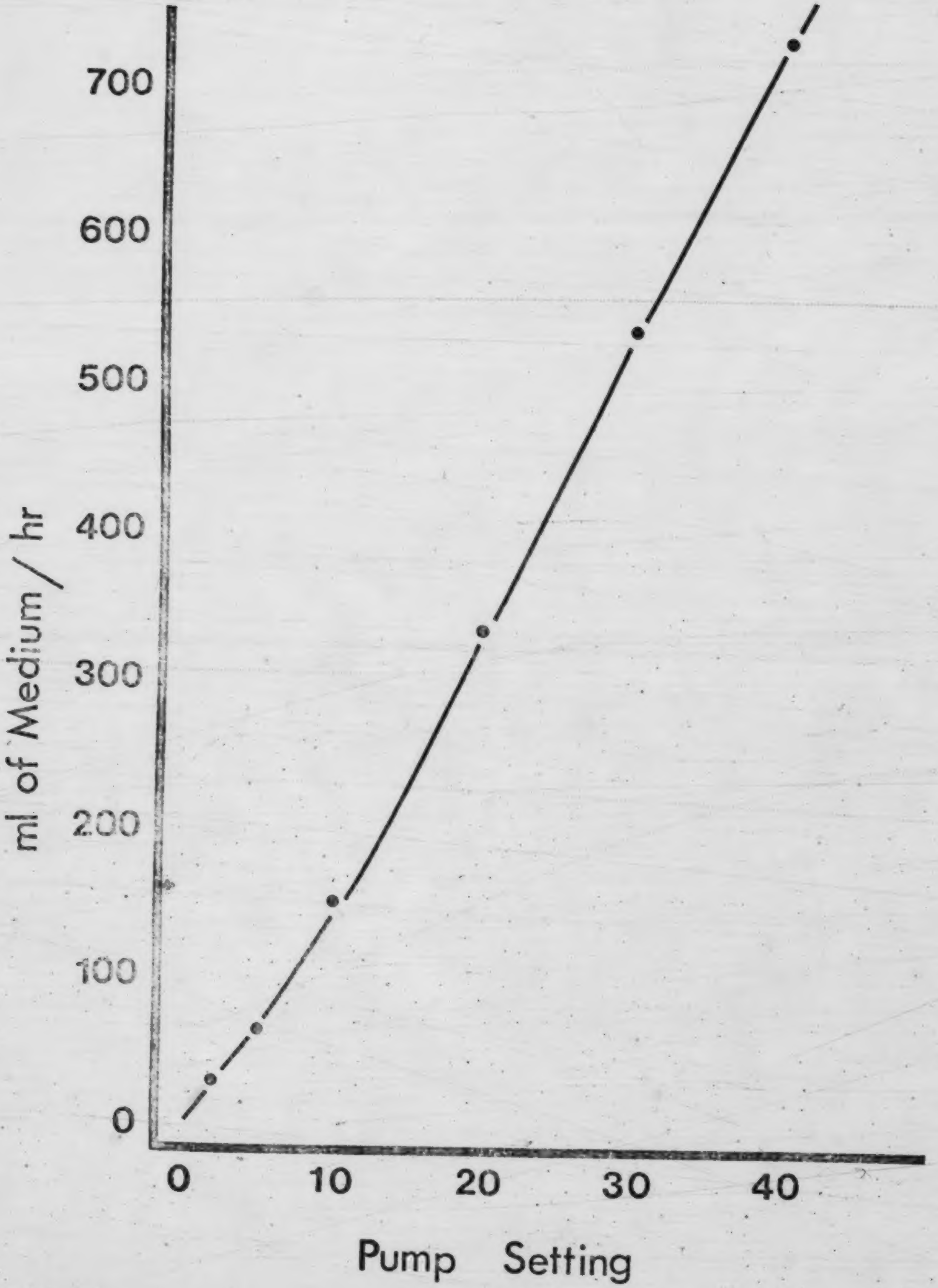
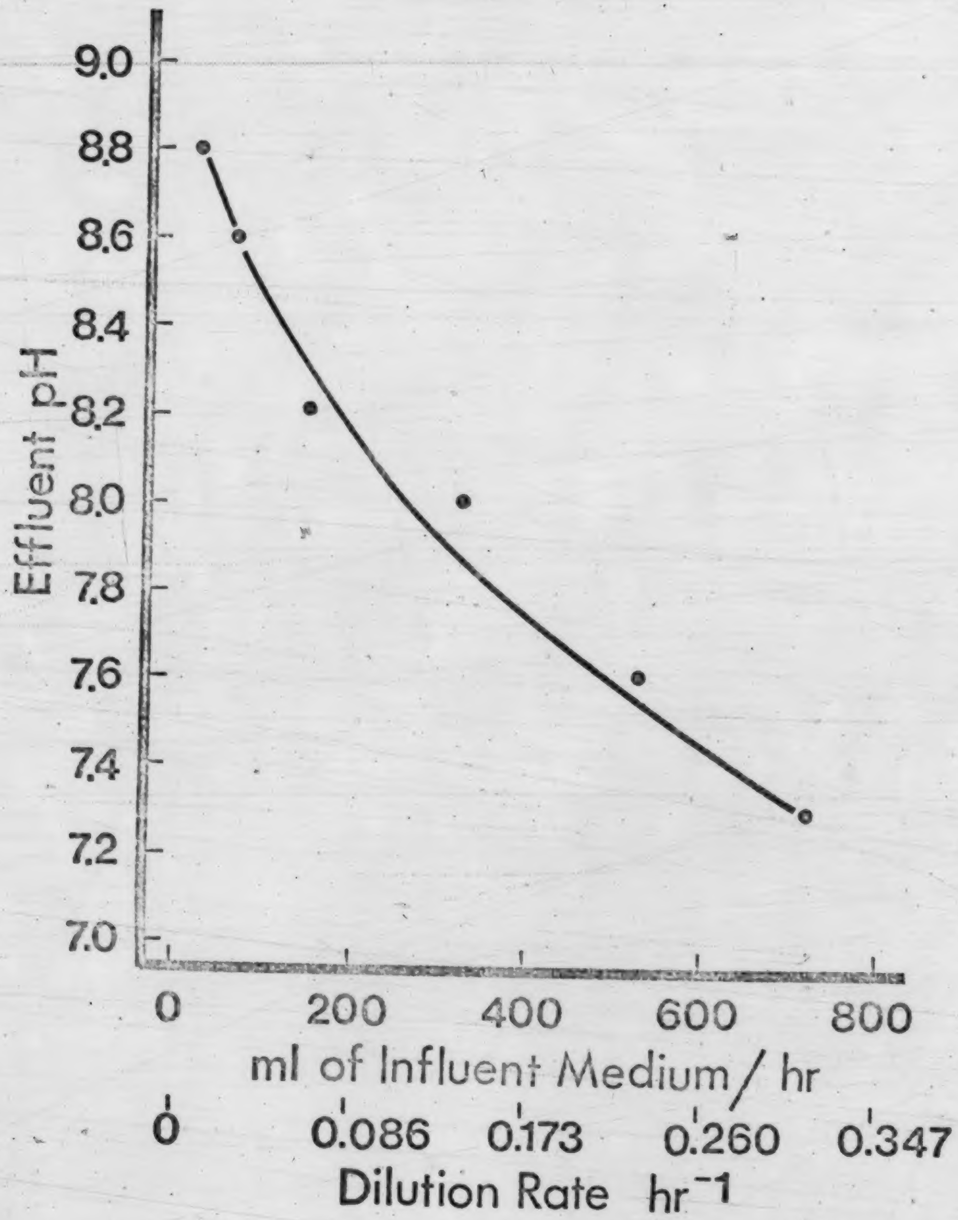

700
600
500
400
300
200
100
0
ml of Medium / hr
0
10
20
30
40
Pump Setting

Figure 7

pH OF THE CHEMOSTAT EFFLUENT FLOW

AS A FUNCTION OF DILUTION RATE


Effluent pH
9.0
8.8
8.6
8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
0
200
400
600
800
ml of Influent Medium/ hr
0
0.086
0.173
0.260
0.347
Dilution Rate hr^{-1}

by the production and liberation of H_2S from the system. It is to be noted that only in the event that H_2S remains trapped in a system will growth by a dissimilatory sulfate reducer have the potential of creating an environment with a weak net acidic reaction.

B. Growth of Isolate #1 as Measured by Viable Counts and the Production of Protein, RNA, and DNA as a Function of Chemostat Dilution Rate

As in the previously-described chemostat experiments, evidence for growth in this study was based on production of cellular protein, D-ribose (RNA), deoxy-D-ribose (DNA), and estimations of viable counts.

(i) Viable Counts

The results of viable count estimations as a function of dilution rate appear in Figure 8. As with all subsequent data in this section, results are presented both on a /ml of effluent and on a /hr production basis. The /ml presentation describes the instantaneous production of a chemostat and is a reflection of the intensity of growth under a particular set of conditions. The /hr production rates illustrate the net production (productivity) /unit of time. The viable count /ml maximized at a dilution rate of 0.065 hr^{-1} while total production of viable cells on an hourly basis reached a maximum at a dilution rate of 0.145 hr^{-1}.

(ii) Protein Production

The instantaneous /ml production of cellular protein as shown in Figure 9 indicates a net decrease at all dilution rates, after a rate of 0.066 hr^{-1} had been achieved. This indicates less intensive growth beyond this point. However, the /hr production rate shows that net

FIGURE 8

VIABLE COUNT PER MILLILITER OF CULTURE EFFLUENT AND TOTAL VIABLE COUNT PER HOUR AS A FUNCTION OF DILUTION RATE

Viable count per ml of culture effluent: (●——●)

Total viable count per hour: (▲............▲)

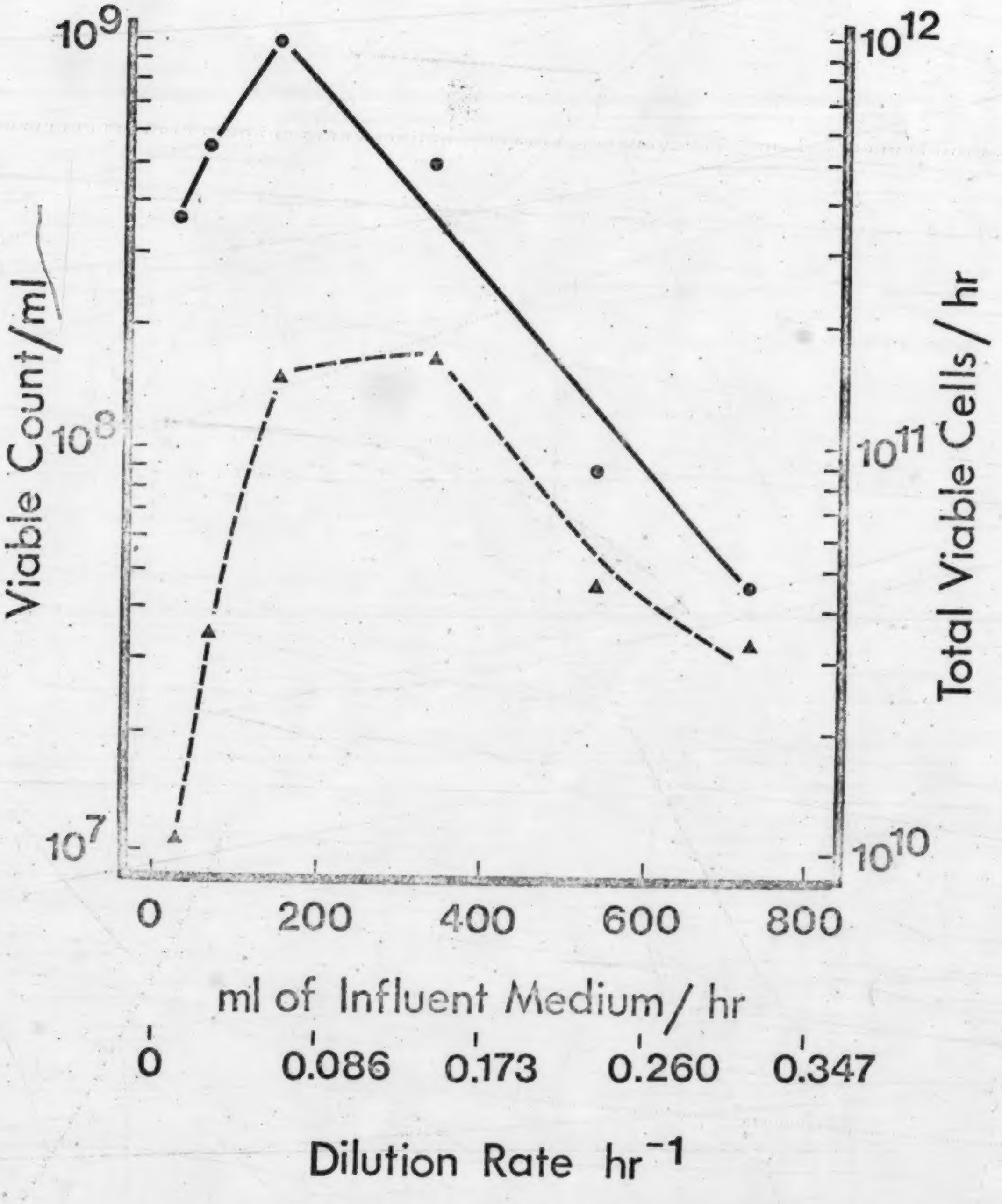

10^9
10^8
10^7
Viable Count/ml
10^12
10^11
10^10
Total Viable Cells / hr
0
200
400
600
800
ml of Influent Medium / hr
0
0.086
0.173
0.260
0.347
Dilution Rate hr^-1

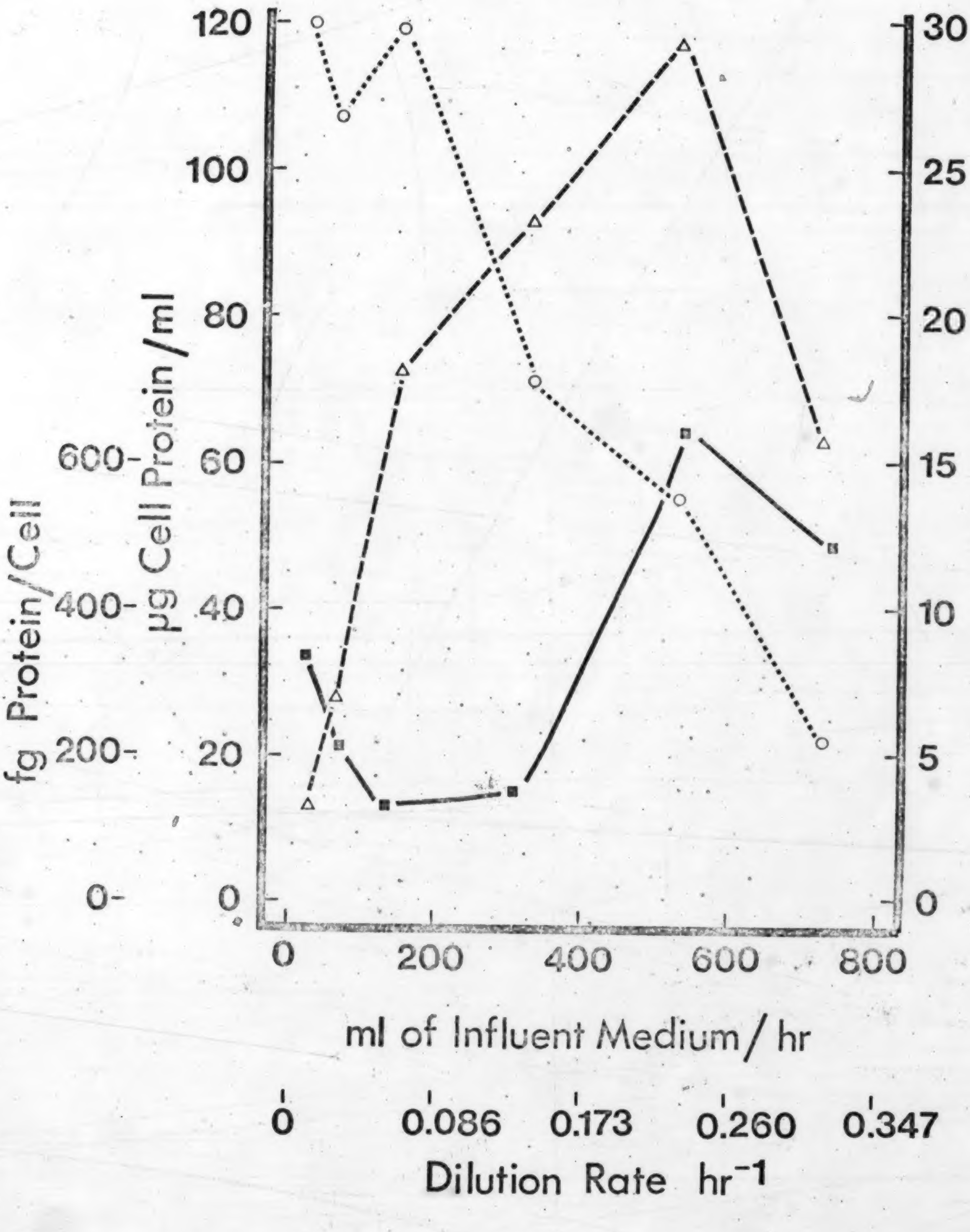
FIGURE 9

CELLULAR PROTEIN PER MILLILITER, PER HOUR, AND PER VIABLE CELL AS A FUNCTION OF DILUTION RATE

Micrograms of cell protein/ml: (○··········○)

Total milligrams of cell protein/hr: (△— — — —·△)

Femtograms of cell protein/viable cell: (■————■)


fg Protein/Cell
0-
200-
400-
600-
µg Cell Protein /ml
0
20
40
60
80
100
120
mg Cell Protein / hr
0
5
10
15
20
25
30
0
200
400
600
800
ml of Influent Medium / hr
0
0.086
0.173
0.260
0.347
Dilution Rate hr⁻¹

cellular protein production was not in fact decreasing until a dilution rate of 0.232 hr^{-1} had been achieved. Beyond this point, unknown factors were causing growth to become suboptimal with respect to cellular protein production. This does not mean that exponential washout was beginning to occur after a dilution rate of 0.232 hr^{-1}, since all dilution rates used in this study were maintained long enough to ensure that any conditions leading to $D > \mu$ would be manifested as washout. This observation simply illustrates that, as pointed out by Hough and Wase (1966), actual growth data can deviate from theoretical production curves noted in many discussions of chemostat kinetics. They state that low protein production values /hr at low growth rates illustrates high initial requirements for maintenance energy.

The graph of cell protein production/ml showed a temporary depression at a dilution rate of 0.028 hr^{-1} (64 ml influent medium/hr). This observation cannot be readily explained, but the finding was repeatable when the range of dilutions, 0.012, 0.028, and 0.065 hr^{-1} were re-run. Nevertheless, even in this range of dilution rates, overall protein production/hr was showing its most rapid increase. The graph of cellular protein production/hr shows maximum positive slope of 2.77 mg of protein/hr increase per 0.01 hr^{-1} increase in dilution rate, up to a dilution rate of 0.065 hr^{-1}. From this point, until a dilution rate of 0.233 hr^{-1}, further increases of cellular protein production/hr occurred, but at a decelerated rate of 0.675 mg protein/hr increase per 0.01 hr^{-1} increase in dilution rate. The change of slope possibly indicates the gradual increase in concentration of undesirable metabolic end-products, e.g., sulfite and sulfide.

The plot of femtograms of protein/viable cell included in Figure 9 illustrates an interesting trend. Up to a dilution rate of 0.065 hr^{-1}, total viable cells/hr and protein production/hr increased. However, viable cells/ml also increased in this range of dilution rates, whereas protein production/ml tended to remain the same or fall. Thus, protein/viable cell in Figure 9 illustrates a negative slope, which is indicative of more cells containing less protein per individual cell. Beyond a dilution rate of 0.065 hr^{-1}, viable counts/ml and total viable cells/hr showed a steady decrease. Nevertheless, protein production/hr still increased, but at a slower rate, until a dilution rate of 0.233 hr^{-1} had been achieved. As can be seen in Figure 9, the protein content/viable cell in this range levelled out and then rose sharply at a dilution rate of 0.233 hr^{-1}. Therefore, in this range, a smaller number of cells were maintaining a higher protein content, the converse of that seen at lower dilution rates. When a dilution rate of 0.316 hr^{-1} was achieved, large decreases in protein/ml, protein/hr, viable counts/ml and /hr, and protein/viable cell occurred (see Figures 8 and 9). The decrease in protein content might suggest that exponential washout had occurred; however, this was not the case, in that growth even at this high dilution rate was stabilized for a significant length of time before the measurements were taken. Indeed, if the growth rate had been exceeded by the dilution rate at the level of influent flow, exponential washout would have been very evident. It would appear that the bacterium could achieve and maintain this growth rate but was limited in its development. It is possible that under such rapid diluting conditions, poising of the medium was likely far from optimal or there may well have been accumulation of inhibiting intermediary metabolites under these sub-optimal conditions. Therefore, while stabilization did occur at this high dilution rate, the chemostat was supporting less total production both in terms of cell protein and viable organisms.

(iii) RNA Production

Figure 10 shows D-ribose production/ml, /hr, and RNA/cell, expressed as a function of dilution rate. As would be expected, there were many similarities between the kinetics of RNA and protein production in cells grown under these conditions. However, there were some important differences noted. Although a temporary loss in /ml production of RNA occurred at a dilution rate of 0.027 hr^{-1}, as was observed with protein, the rate of increase in /ml production of RNA during dilution rate increases up to 0.065 hr^{-1} was far greater than the corresponding increase in protein production/ml. In terms of /hour production, the increase in RNA as a function of increasing dilution was constant to a dilution rate of 0.233 hr^{-1} with no apparent change in rate. This too differs from /hour production of protein where at least one rate change was observed. It would appear that this Desulfovibrio species in continuous culture, follows the same trend observed by Kjeldgaard (1967) with Salmonella typhimurium, namely as the cellular growth rate increases, RNA levels increase more rapidly than do protein levels (on a /hr production basis). The /ml production of RNA decreased with increasing flow or dilution rate after 0.065 hr^{-1} as did cellular protein concentration; but the curve shows a less negative slope in the case of RNA. Figure 10 also presents the RNA levels/cell as a function of dilution rate. As with the protein levels/cell described previously, RNA levels/cell decreased to a plateau level at a dilution

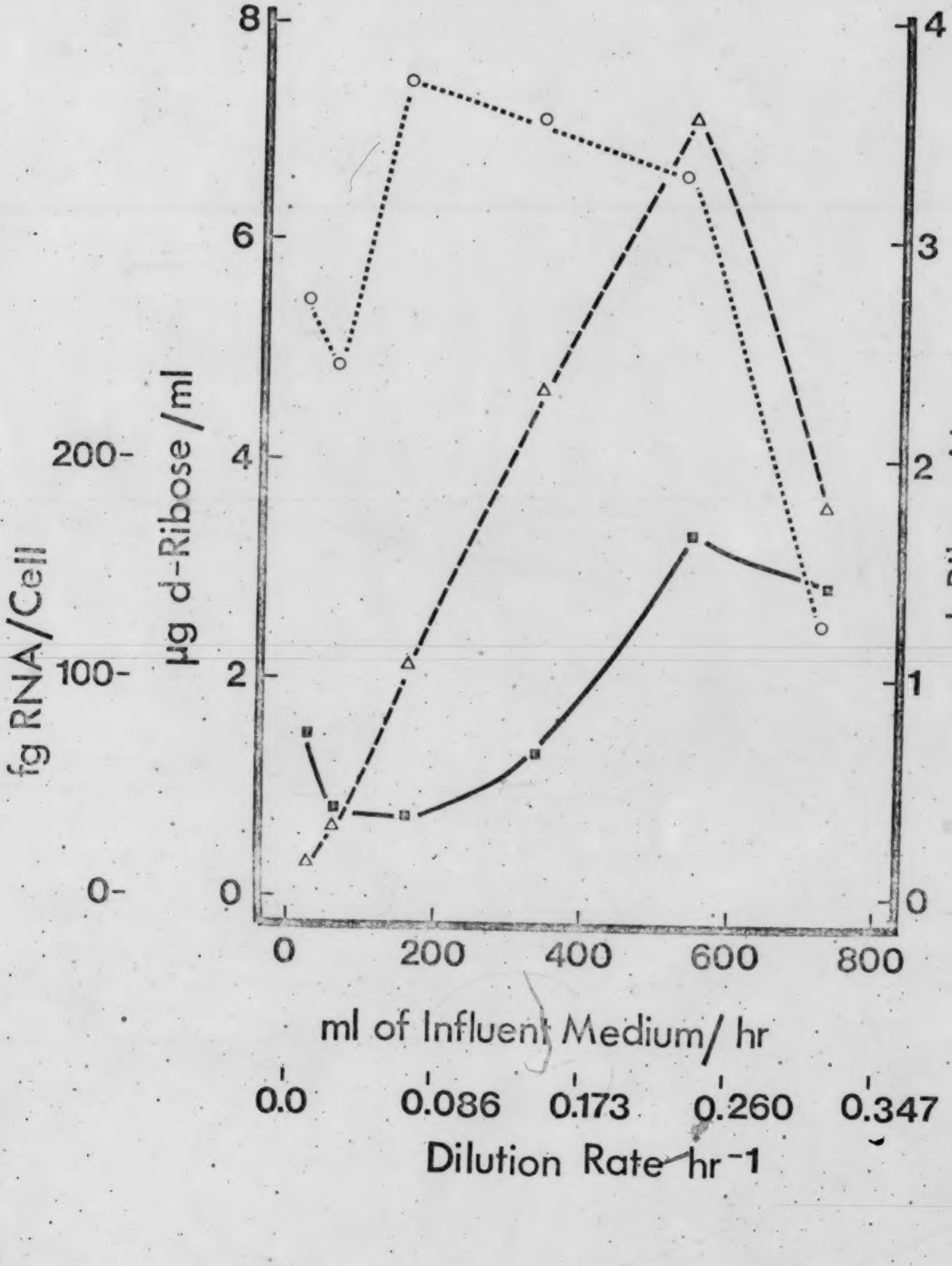
FIGURE 10

RNA-RIBOSE PER MILLILITER AND PER HOUR AND RNA PER VIABLE CELL AS A FUNCTION OF DILUTION RATE

Micrograms of D-ribose/milliliter of culture effluent: (○··········○)

Milligrams of D-ribose produced/hour: (△– – – – –△)

Femtograms of RNA/cell: (■———■)


fg RNA/Cell
0-
100-
200-
µg d-Ribose /ml
0
2
4
6
8
mg d-Ribose/ hr
0
1
2
3
4
0
200
400
600
800
ml of Influent Medium/ hr
0.0
0.086
0.173
0.260
0.347
Dilution Rate hr -1

rate of 0.065 hr^{-1}, and then increased to maximum at a dilution rate of 0.233 hr^{-1}, before decreasing once again at the highest growth rate (0.316 hr^{-1}) used. RNA production, both /ml and /hr, showed a rapid decrease beyond a dilution rate of 0.233 hr^{-1}. Again this should be regarded as a physiological manifestation of growth under suboptimal conditions.

(iv) DNA Production

The production of DNA by Isolate #1 in continuous culture, both /ml and /hr as a function of dilution rate, is shown in Figure 11. The kinetics of DNA production under these conditions differed greatly from both protein and RNA production. From a dilution rate of 0.012 to one of 0.065 hr^{-1}, the instantaneous /ml production increased very rapidly, with a depression at the intermediate dilution rate, 0.027 hr^{-1}. This very rapid increase in /ml production of DNA would indicate growth conditions becoming more optimal with increasing dilution rate and therefore growth rate. This does not mean that /viable cell, the content of DNA was increasing. In fact, Figure 11 shows that the concentration of DNA/viable cell was decreasing in the range where the content/ml was showing the most rapid increase. This would imply that the cells were maintaining fewer copies of genome/viable unit as growth rates increased. Because the levels of DNA increased with increasing flow rate, the /hr production rates naturally increased very rapidly. At dilution rates greater than 0.065 hr^{-1}, per hour production rates of DNA fell slowly until a dilution rate of 0.233 hr^{-1} was reached, where a rapid decrease occurred. This of course showed up as a very rapid decrease in DNA levels on a per ml basis. It would appear that with increasing dilution rate, the per hour production rates of protein and

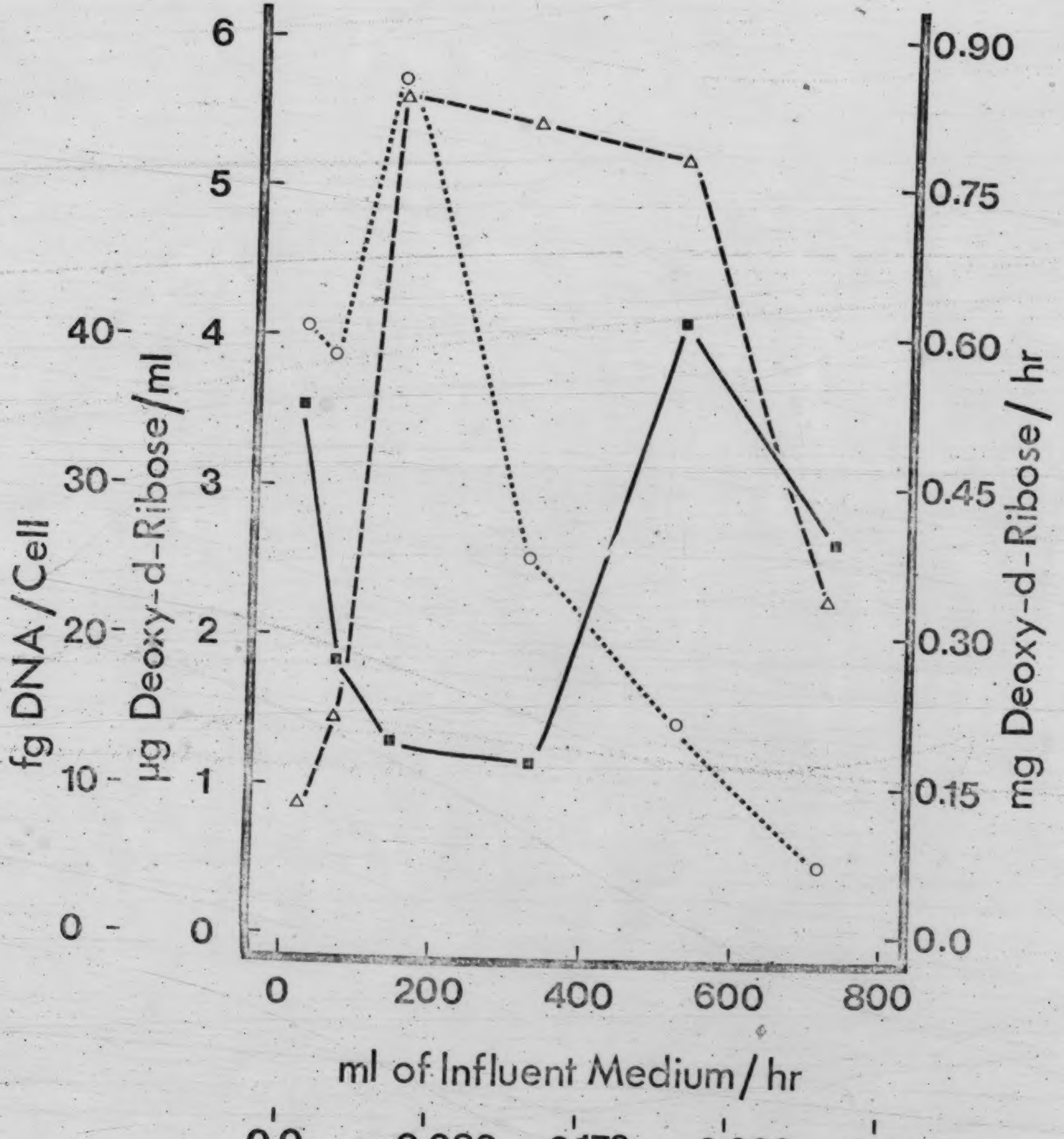
FIGURE 11

DEOXY D-RIBOSE PER MILLILITER AND PER HOUR AND DNA PER VIABLE CELL AS A FUNCTION OF DILUTION RATE

Micrograms of deoxy D-ribose/ml of culture effluent: (○··········○)

Milligrams of deoxy D-ribose produced/hour: (△------△)

Femtograms of DNA/viable cell: (■——■)


fg DNA / Cell
µg Deoxy-d-Ribose / ml
mg Deoxy-d-Ribose / hr
0
10
20
30
40
0
1
2
3
4
5
6
0.0
0.15
0.30
0.45
0.60
0.75
0.90
0
200
400
600
800
ml of Influent Medium / hr
0.0
0.086
0.173
0.260
0.347
Dilution Rate hr^{-1}

nucleic acid decreased in the following order: DNA > protein > RNA.

Figure 12 summarizes graphically the protein:RNA, protein:DNA, and RNA:DNA ratios, as a function of dilution rate. Since the protein:RNA ratios (Figure 12) show a negative slope which levels off at dilution rates around 0.145 hr^{-1}, the rate of RNA synthesis rose faster than did protein synthesis throughout the dilution rates tested. At low dilution rates, i.e., rates of 0.028 hr^{-1}, the synthesis of DNA apparently occurred at a faster rate in relative terms than did protein synthesis. At dilution rates greater than 0.065 hr^{-1}, the slope of the protein:DNA ratio increased steadily. This indicates that rates of DNA synthesis were falling behind protein synthesis at an increasing rate. It would seem that of the three main physiological parameters measured, DNA synthesis seemed to show the least amount of control during the testing of the various growth rates. This is based on the observation that DNA levels varied erratically at low dilution rates and decreased earlier and faster than either protein or RNA levels (compare Figure 9, 10, and 11). The fact that the RNA:DNA curve showed a positive slope as the growth rate was increased indicated that RNA content was increasing more rapidly than DNA content.

The ratio curves (Figure 12) indicate that this organism obeys one of the general rules of increasing growth rates, i.e., that RNA synthesis rates increase first. It is apparent that DNA synthesis is not, however, under ideal or tight control. It is especially obvious that under very low dilution rates, e.g., 0.012 hr^{-1}, the cells managed all their physiological functions with a minimum concentration of RNA being present. In fact, at this growth rate, there was more DNA than RNA present in the cells. Even at a dilution rate of 0.065 hr^{-1},

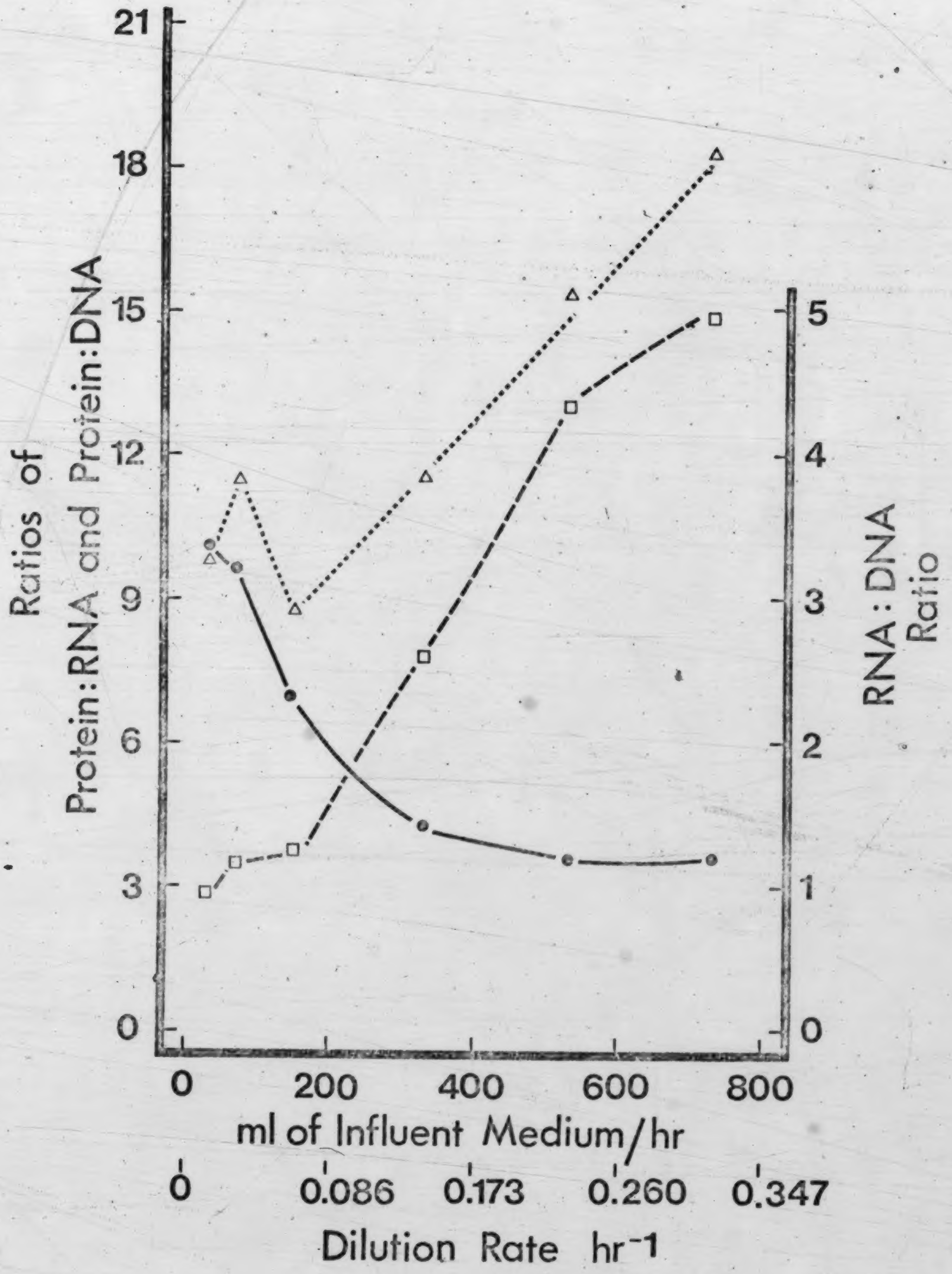
FIGURE 12

PROTEIN:RNA, PROTEIN:DNA AND RNA:DNA RATIOS AS A FUNCTION OF DILUTION RATE

Protein:RNA ratio: (●————●)

Protein:DNA ratio: (△·················△)

RNA:DNA ratio: (□– – – – – – – –□)


Ratios of
Protein:RNA and Protein:DNA
0
3
6
9
12
15
18
21
RNA : DNA
Ratio
0
1
2
3
4
5
0
200
400
600
800
ml of Influent Medium/hr
0
0.086
0.173
0.260
0.347
Dilution Rate hr-1

DNA levels were still only slightly below those of RNA. Throughout this and the previous chemostat experiment, it appears that under certain conditions, e.g., slow growth rates, these cells contain an inordinantly large amount of DNA in relation to their protein and RNA levels.

C. Cell Size Distribution of Isolate #1 as a Function of Dilution Rate

Cell size distribution studies were undertaken in the same manner as described in the previous chemostat study. Data are presented in detail in Table XVII, and graphically in Figure 13. It should be noted that size distribution analysis is not available for the most rapid dilution rate (0.316 hr^{-1}). The data presented do show a significant trend in size distribution patterns. In the dilution rate range of 0.012-0.065 hr^{-1}, there was wide variation in cell size with distribution peaks at 2.4 μm, 3.6 μm or both. This indicates a greater proportion of large cells in the population. Dilution rates beyond 0.145 hr^{-1} produced cell populations more uniform in size with a sharp frequency peak occurring at 2.5 μm, and with no cells showing lengths greater than 4.2 μm. In addition, the frequency of cells in the range of 1.2 to approximately 2.8 μm in length was much greater than was observed at lower dilution rates. This indicates that fast growth was yielding smaller cells, even though as shown previously (Figure 9), these cells contained a higher protein content per individual cell.

D. The Production of Intermediary Metabolites and End-Products of Dissimilatory Sulfate Reduction as a Function of Dilution Rate

The concentrations of sulfide, sulfite, and thiosulfate in solution,

TABLE XVII. SUMMARY OF CELL SIZE DISTRIBUTION AND HALF-CYCLE FREQUENCY AS A FUNCTION OF CHEMOSTAT DILUTION RATE

Cell Length (μm)	Number of 1/2 cycles	Percent of Total Population Dilution Rate (hr^{-1}) 0.012	0.027	0.065	0.145	0.233
1.2	1	3.0	0.0	0.0	0.0	0.0
		$\Sigma 3.0$				
1.8	1	4.2	2.4	1.2	27.0	24.0
	2	3.6	1.8	5.5	0.0	0.6
		$\Sigma 7.8$	4.2	6.7	27.0	24.6
2.4	1	9.8	8.5	1.8	26.0	31.0
	2	22.0	18.4	18.5	15.9	15.0
	3	4.9	5.5	6.7	0.0	0.0
		$\Sigma 36.7$	32.4	27.0	41.9	36.0
3.0	1	0.0	1.8	0.0	1.5	1.0
	2	20.2	12.2	11.7	17.5	18.7
	3	5.5	9.2	12.9	0.5	0.0
	4	0.0	0.0	2.4	0.0	0.0
		$\Sigma 25.7$	23.2	27.0	19.5	19.7
3.6	2	6.1	4.2	0.6	9.5	6.8
	3	9.2	19.6	15.4	1.6	1.8
	4	1.2	1.8	7.4	0.0	0.0
		$\Sigma 16.5$	25.6	23.4	11.1	8.6
4.2	2	1.2	1.2	0.0	0.0	0.0
	3	6.1	5.5	4.3	0.0	0.0
	4	0.6	3.0	8.0	0.0	0.0
		$\Sigma 7.9$	9.7	12.3		
4.8	3	1.8	1.2	1.2	0.0	0.0
	4	0.0	3.6	1.8	0.0	0.0
		$\Sigma 1.8$	4.8	3.0		

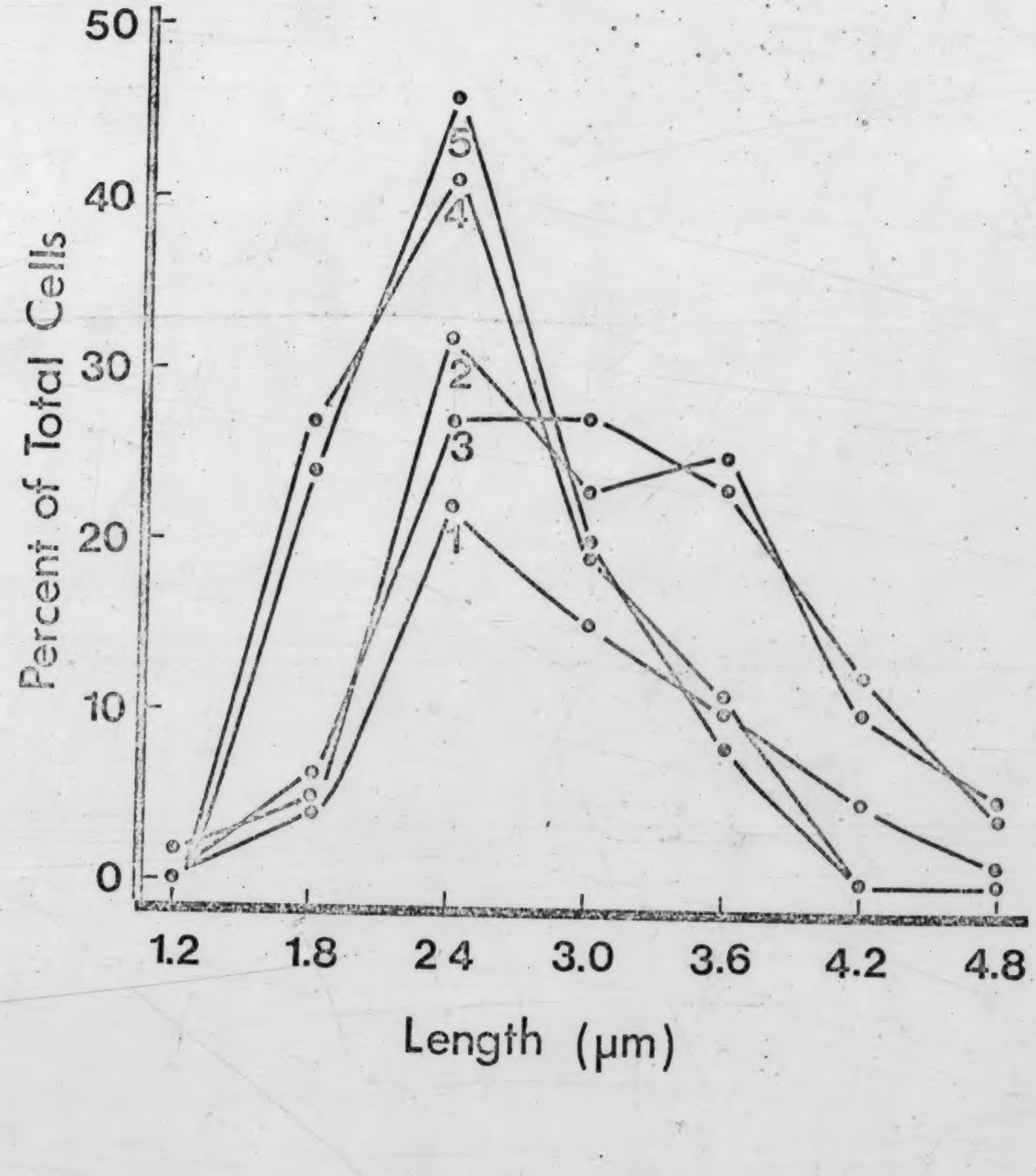
Figure 13

SIZE DISTRIBUTION OF ISOLATE #1 CELLS

AS A FUNCTION OF DILUTION RATE

All plotted points are the summed values for each measured length obtained from Table XVII.

Plot Number	Influent Flow Rate (ml/hr)	Dilution Rate
1	28	0.012 hr^{-1}
2	64	0.027 hr^{-1}
3	151	0.065 hr^{-1}
4	333	0.145 hr^{-1}
5	536	0.233 hr^{-1}


50
40
30
20
10
0
Percent of Total Cells
5
4
2
3
1
1.2
1.8
2.4
3.0
3.6
4.2
4.8
Length (μm)

and effluent H_2S were determined by the methods outlined in the Materials and Methods section.

(i) Solution and Sparged Sulfide Levels

The concentration/ml of solution sulfide and total production/hr as a function of dilution rate is presented in Figure 14. The /ml production of sulfide reached a maximum at a dilution rate of 0.028 hr^{-1}. Thus, at this growth rate, cells in the chemostat were exposed to their highest levels of sulfide (5.35 μmoles/ml). This does not include gaseous H_2S which was continuously being sparged from the system (408 μmoles/hr or 0.113 μmoles/second; see Figure 15). This high concentration of sulfide corresponds to and may well have been the cause of depressed levels of RNA, DNA, and protein which occurred at this dilution rate. The /hr production of solution sulfide reached a maximum at a dilution rate of 0.145 hr^{-1}, which corresponds very well to the maximum/hr production of sparged H_2S seen in Figure 15. The only difference in these two curves is that sparged H_2S spiked sharply at this growth rate, while a smoother curve describes the /hr solution sulfide production. The decrease in sulfide and H_2S production beyond a dilution rate of 0.145 hr^{-1} indicates that the chemostat was less toxic, but was also a weaker reducing system. Because of this, poising was probably not ideal, and as can be seen from previous figures, growth of cells in the chemostat decreased. This would have a multiplier effect, since the cells, in a stress situation, would in turn respire at an even more depressed level, leading to less H_2S production and hence a further reduction in self-poising capabilities.

FIGURE 14

MICROMOLES PER MILLILITER OF EFFLUENT AND TOTAL PRODUCTION PER HOUR OF SOLUTION SULFIDE AS A FUNCTION OF DILUTION RATE

Micromoles of solution sulfide/ml of effluent: (●———●)

Micromoles of solution sulfide produced/hour: (▲———▲)

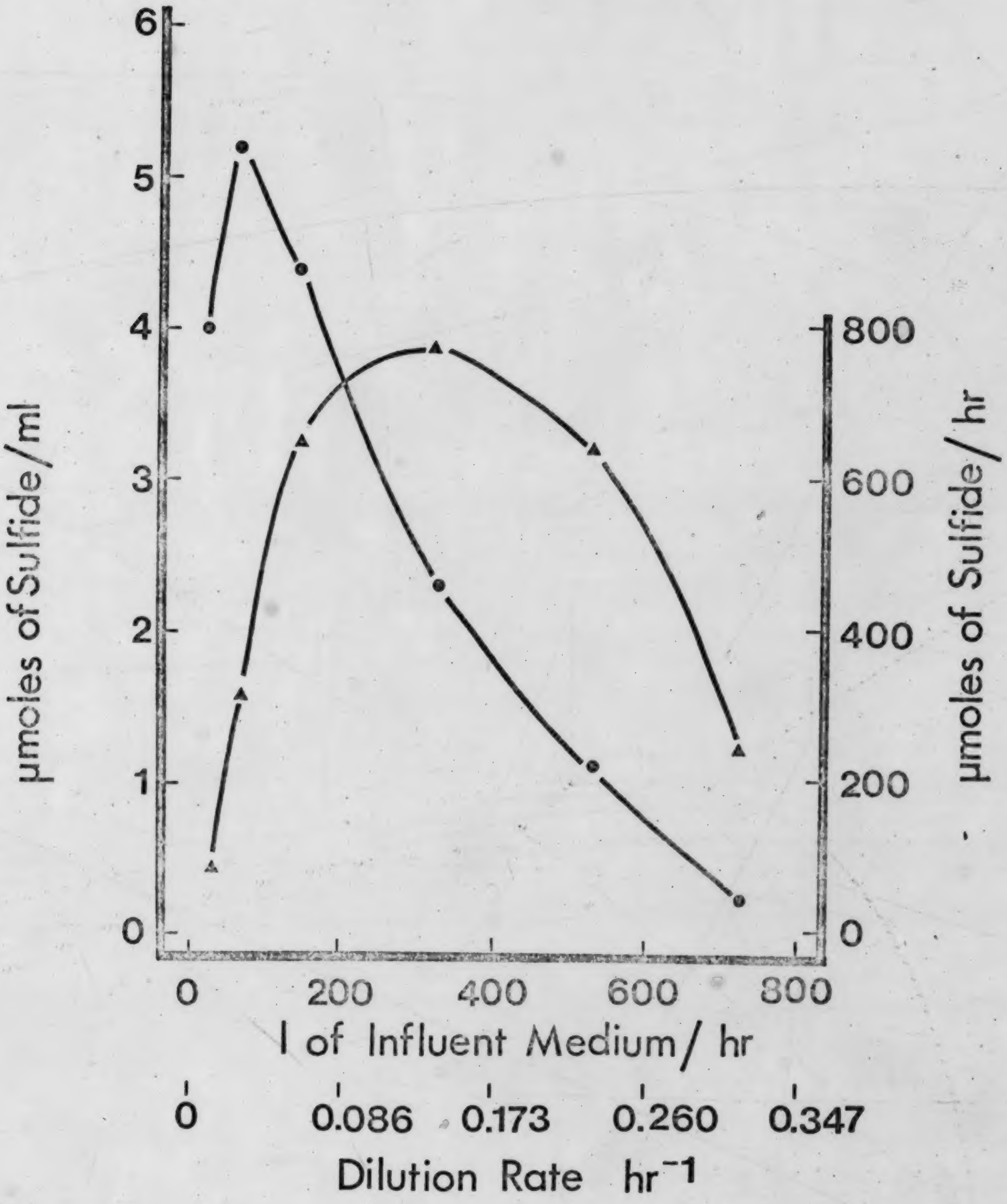
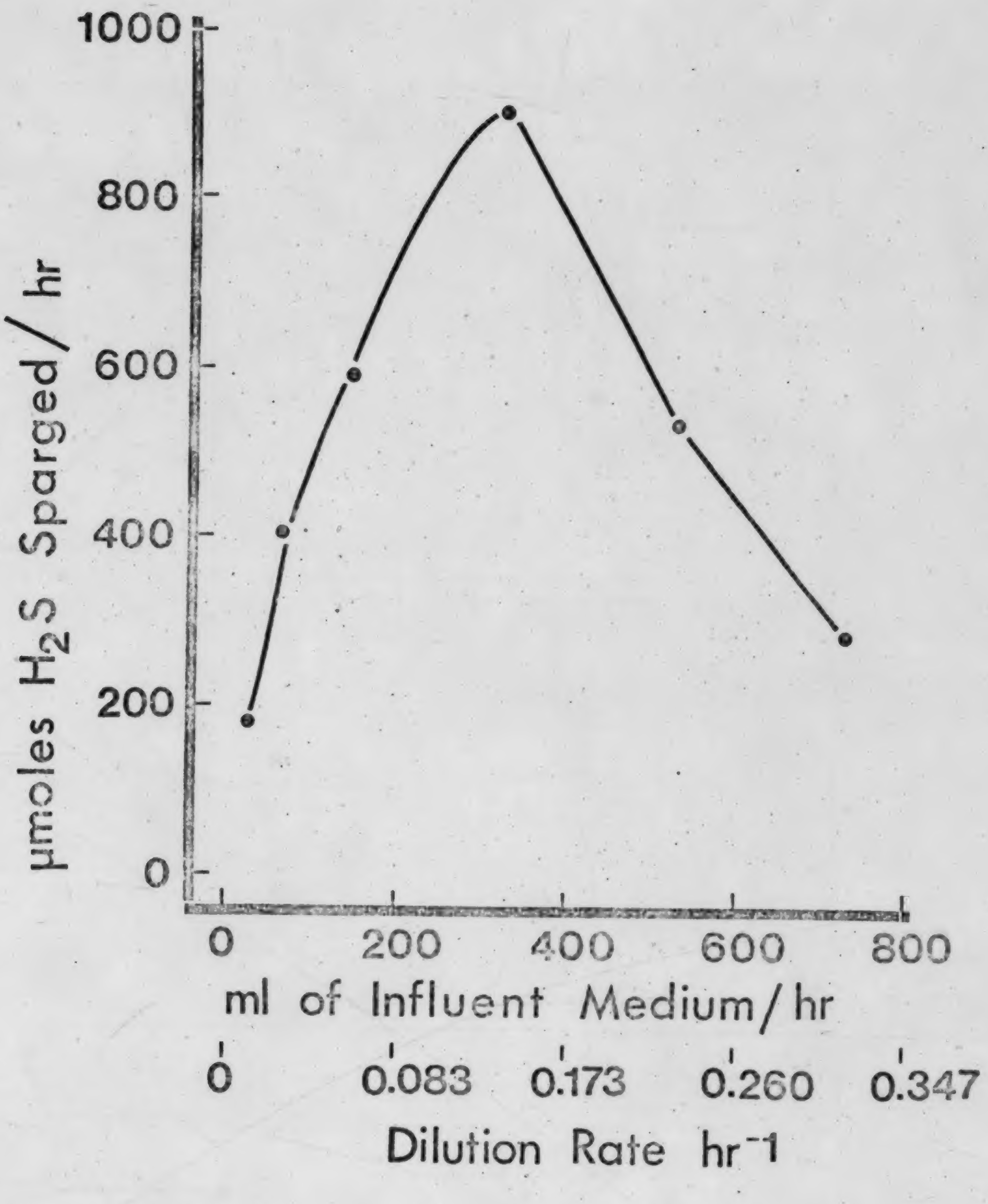

µmoles of Sulfide/ml
0
1
2
3
4
5
6
µmoles of Sulfide/hr
0
200
400
600
800
0
200
400
600
800
l of Influent Medium/ hr
0
0.086
0.173
0.260
0.347
Dilution Rate hr⁻¹

Figure 15

MICROMOLES OF HYDROGEN SULFIDE SPARGED FROM THE CHEMOSTAT SYSTEM AS A FUNCTION OF DILUTION RATE


1000
800
600
400
200
0
µmoles H_2S Sparged / hr
0
200
400
600
800
ml of Influent Medium / hr
0
0.083
0.173
0.260
0.347
Dilution Rate hr^{-1}

(ii) Sulfite Production

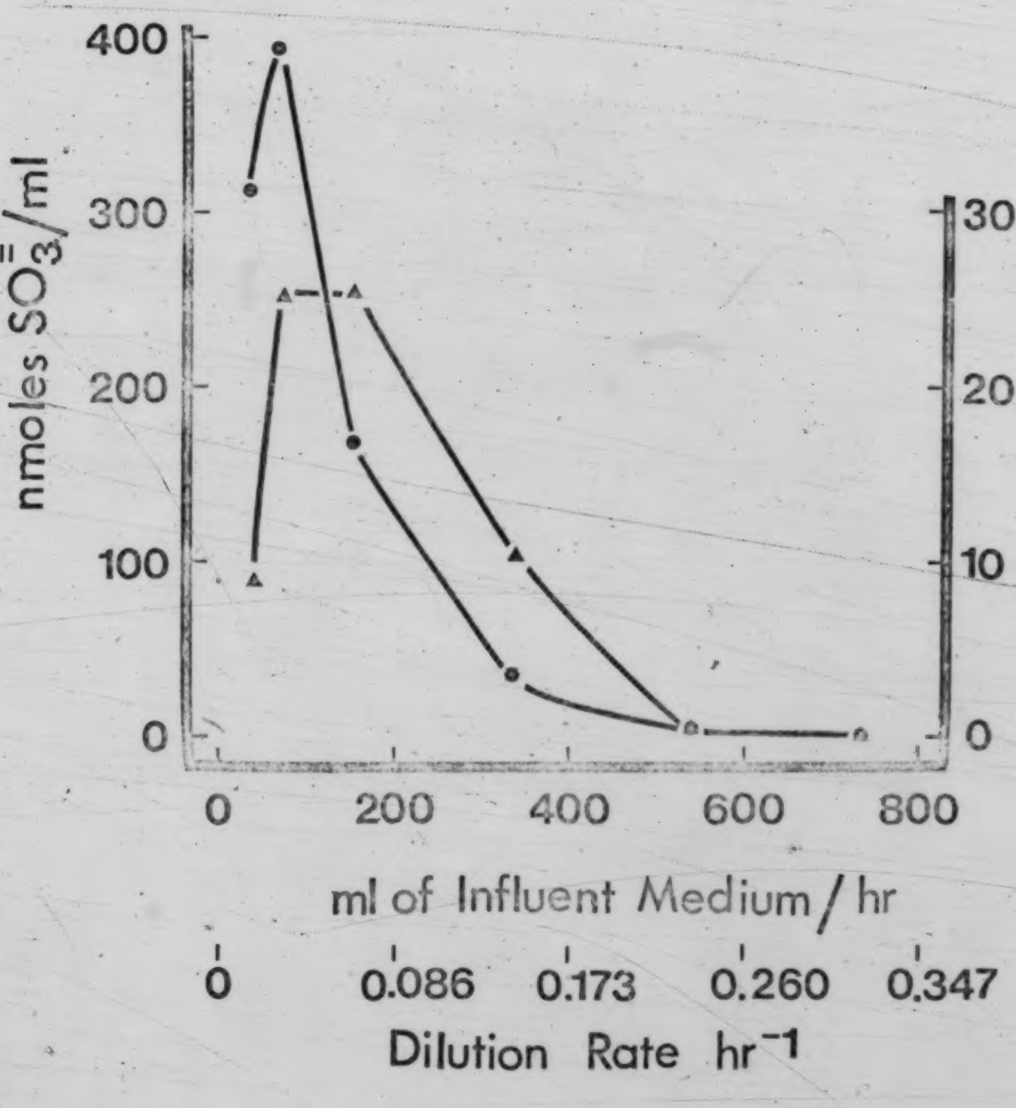
Figure 16 represents sulfite production /ml and /hr as a function of dilution rate. The effluent levels of sulfite and thiosulfate were followed closely in this study since some of the dilution rates tested could lead to unbalanced growth, and the accumulation of reduced forms of sulfur. The graphs of sulfite production/ml and /hr show maximum accumulation of sulfite between dilution rates 0.027 and 0.065 hr^{-1}. However, this cannot be considered a manifestation of unbalanced growth, since this was in a region of maximum cellular production. It is interesting, however, to note that like solution levels of sulfide, the /ml concentration of sulfite maximized at a dilution rate of 0.027 hr^{-1}. Butlin (1949) stated that the presence of sulfite in Desulfovibrio cultures would encourage the development of grossly enlongated cells; but more important, it rapidly became toxic even to cultures adapted to use it as the primary electron acceptor. It is doubtful that a $SO_3^=$ concentration of 0.39 μmoles/ml would prove toxic to this culture; but it could be slightly inhibitory to certain cellular processes, which again might correspond to the temporary depression of the instantaneous /ml production levels of RNA, DNA, and protein. The most important observation gained from following sulfite levels was that at high dilution or growth rates, sulfite as an intermediate did not accumulate. Significant effluent concentrations of sulfite could not be detected beyond a growth rate of 0.233 hr^{-1}. Such an observation might be explained as follows. At lower dilution rates, significant concentrations of sulfide are present in the culture medium which could in fact exert some end-product repression against certain enzymes present earlier in the

FIGURE 16

NANOMOLES OF SULFITE PER MILLILITER
OF CHEMOSTAT EFFLUENT AND TOTAL
MICROMOLES OF SULFITE PER HOUR AS
A FUNCTION OF DILUTION RATE

Nanomoles of sulfite/ml of effluent: (●———●)

Total micromoles of sulfite/hr: (▲———▲)



400
300
200
100
0
nmoles $SO_3^=$/ml
30
20
10
0
μmoles $SO_3^=$/hr
0
200
400
600
800
ml of Influent Medium/hr
0
0.086
0.173
0.260
0.347
Dilution Rate hr^{-1}

dissimilatory pathway, e.g., bisulfite reductase. Thus, sulfite accumulated as a result of sulfate activation during the low dilution rates tested. At higher dilution rates, less sulfide was present, therefore end-product repression would probably not be a problem. Any sulfite formed could then be fully reduced to sulfide, or partially reduced to the level of thiosulfate or trithionate (as per the equations listed in the Literature Review).

(iii) Thiosulfate Production.

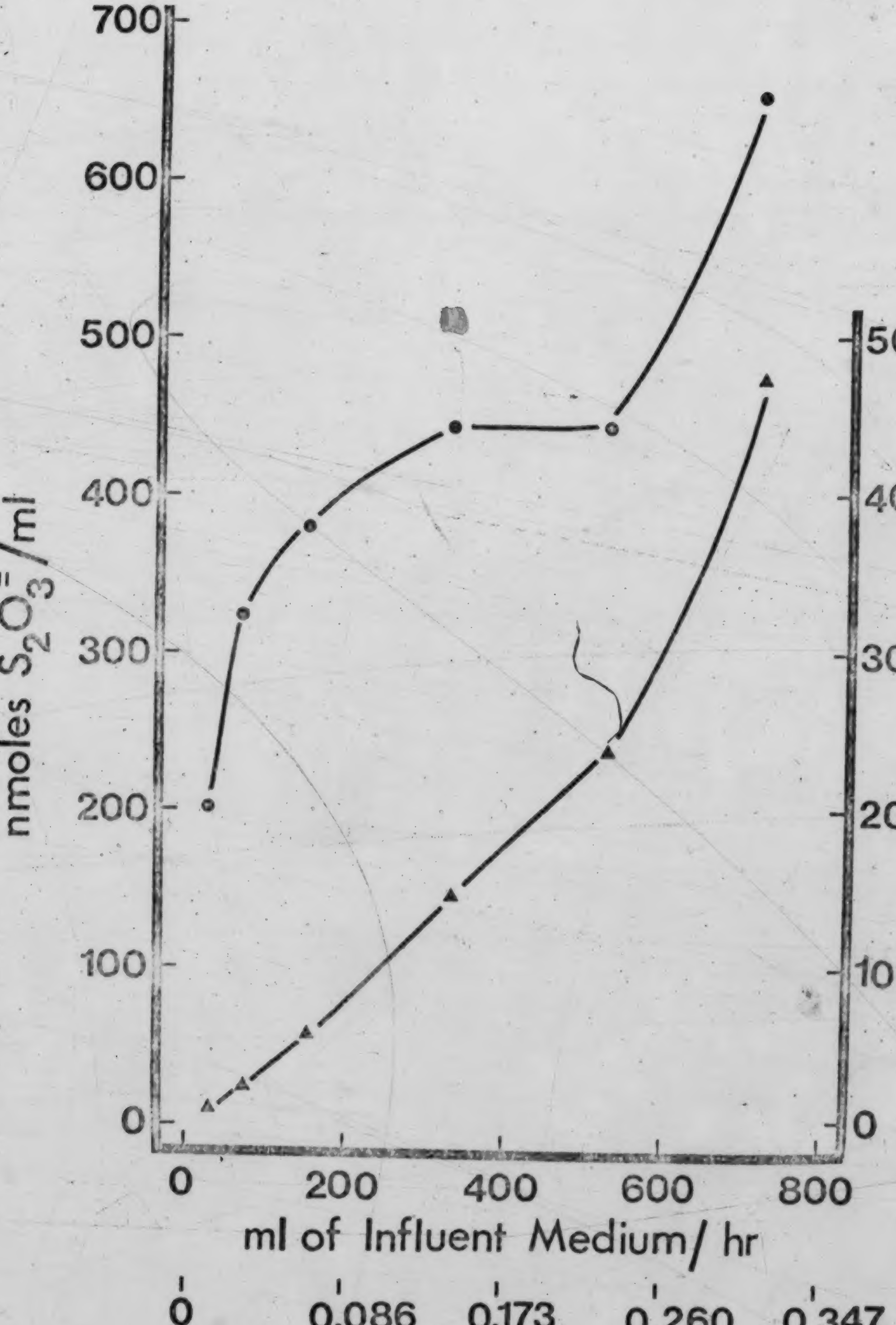
The only other intermediate of dissimilatory sulfate reduction assayed for was thiosulfate. Its production /ml and /hr is shown graphically in Figure 17. The /ml production curve shows clearly that thiosulfate accumulation occurred at differing rates during the various dilution rates tested. For example, the transition from a dilution rate of 0.233 to 0.316 hr^{-1} resulted in a significant increase in per hour accumulation of thiosulfate. Unlike the eventual lack of accumulation of sulfite at higher dilution rates, thiosulfate levels follow a steady increase as the dilution rate increases. It appears from this data that the final reductive step involving the conversion of thiosulfate to sulfide and sulfite is a rate-limiting step and under conditions of rapid growth, e.g., high dilution rates, thiosulfate accumulates. If taken far enough, this could result in the shutdown of the reduction of trithionate to thiosulfate and sulfite. This would not cause an accumulation of sulfite since sulfite apparently recycles into synthesis of trithionate. Thus thiosulfate would accumulate first (assuming the rate-limiting step occurs at the point of thiosulfate reduction) and trithionate would not accumulate until growth conditions were even more unbalanced.

FIGURE 17

NANOMOLES OF THIOSULFATE PER MILLILITER OF CULTURE EFFLUENT AND TOTAL MICROMOLES OF THIOSULFATE PRODUCED PER HOUR AS A FUNCTION OF DILUTION RATE

Nanomoles of thiosulfate/ml of effluent: (●————●)

Total micromoles of thiosulfate/hr: (▲————▲)


700
600
500
400
300
200
100
0
nmoles $S_2O_3^=$/ml
500
400
300
200
100
0
μmoles $S_2O_3^=$/ hr
0
200
400
600
800
ml of Influent Medium/ hr
0
0.086
0.173
0.260
0.347
Dilution Rate hr^{-1}

Senez (1962) speculated that in the pathway of sulfite reduction to sulfide, energy expenditure was required, although he presented no justification for this hypothesis. Thus, there is the possibility that thiosulfate reduction is rate-limiting in the sense that it may require energy. If, in fact, thiosulfate reduction is an endergonic process, unbalanced growth at high dilution rates could lead to a lack of available ATP, a breakdown in thiosulfate reduction, and a consequent increase in thiosulfate concentrations.

E. Sulfur Recoveries

In studies such as reported in this section, it is important to account for both the products and the components used in respiration. There is very little other data in the literature concerning the relationship between sulfate consumption and production of reduced sulfur intermediates. For example, it is not known whether the routes for dissimilatory reduction shown in the Literature Review describe all sulfur intermediates which might appear under different growth conditions. The pathways presented are not concerned with the relative concentrations of sulfite, thiosulfate or thrithionate which one might expect to be found under slow, intermediate, or rapid growth conditions.

Data shown in Table XVIII exhibit a general and interesting trend. The recovery of sulfur increased as the dilution rate increased and eventually reached theoretical for the fastest dilution rates studied. The assay systems used at all these dilution rates were standardized as stipulated in Materials and Methods and there is no reason to suspect a systematic error throughout the study. Rather, these results would tend to indicate that at low dilution rates, many sulfur species, or perhaps only trithionate, accumulated, which would not be determined by

TABLE XVIII. INFLOW AND OUTFLOW RECOVERIES OF SULFUR AS A FUNCTION OF DILUTION RATE

	Dilution Rate (hr^{-1})					
	0.012	0.027	0.065	0.095	0.233	0.316
Inflow of $SO_4^=$ sulfur (μmoles)/hr	428	979	2310	5094	8200	11153
Outflow of $SO_4^=$ sulfur (μmoles)/hr	61.6	115.8	717	2930	6300	9841
Outflow of sulfide sulfur (μmoles)/hr	115	324	680	799	670	269
Outflow of thiosulfate sulfur (μmoles)/hr	11	40	114	293	471	947
Outflow of sulfite sulfur (μmoles)/hr	8.6	24.9	24.9	9.9	0	0
Sulfur as H_2S sparged (μmoles)/hr	188	408	597	909	650	283
Total outflow of sulfur accounted for (μmoles)/hr	384.2	912.7	2132.9	4940.9	8091	11340
% of influent sulfur accounted for	89.9	93.1	92.3	97.0	98.6	101.6

the methods used for assaying sulfate, sulfite, thiosulfate, and sulfide. At high dilution rates, a very straight-forward dissimilatory sulfate reduction route was apparent except for the accumulation of thiosulfate. Alternatively, these recoveries could indicate that trithionate was not being accumulated in any quantities at fast growth rates.

F. Sulfur Isotope Fractionation

The $\delta^{34}S$ values for effluent sulfate samples, as listed in Table XIX, do show a trend; namely a maximizing of fractionation at lower dilution (growth) rates with a decrease in fractionation as the growth rate increased. This is in keeping with the observations of Harrison and Thode (1957) and Roy and Trudinger (1971) who state that maximum fractionation occurs at low growth rates, with excess sulfate present, under suboptimal growth temperatures. Clearly in this experiment, maximum fractionation occurred at lower dilution rates, i.e., the cells preferentially utilized the lighter isotope of sulfate-sulfur, thus leaving the residual sulfur enriched in the heavier isotope. Previous papers by other authors were not specific in terms of how much sulfate in growth media was necessary before it was in excess. Assays in this system showed that even in the case of the lowest dilution rate, some sulfate (2.2. μmoles/ml) remained in the effluent medium. Apparently this was sufficient to still allow the cells to discriminate and allow fractionation to occur. No attempt was made to grow Isolate #1 under a suboptimal growth temperature. It would seem, however, that this is merely a convenient way of lowering the growth rate in batch culture situations. In a chemostat, this is easily accomplished by lowering the dilution rate.

TABLE XIX. δ^{34}S VALUES FOR INFLUENT AND EFFLUENT SULFATE AS A FUNCTION OF DILUTION RATE

Dilution Rate	^{34}S Influent $SO_4^=$	^{34}S Effluent $SO_4^=$
0.012	-0.02	+5.3
0.027	-0.02	+6.9
0.065	-0.02	+4.3
0.145	-0.02	+1.9
0.233	-0.02	-0.06
0.316	-0.02	_0.01

NOTE: Each of the per ml deviation values is the mean from six ratio determinations for a given sample on the mass spectrometer.

The data in Table XIX show that significant fractionation ceased under conditions of rapid cell growth (high dilution or growth rate). It is interesting that under these same conditions, thiosulfate also accumulated at an increasing rate. The accumulation of this intermediate, coupled with decreasing fractionation, would seem to agree with the steady state theory of fractionation proposed by Rees (1973). His theory states that fractionation is the sum of the discrimination between sulfur isotopes which occurs at each enzymatic step of reduction. Since significant thiosulfate accumulation occurred at the higher dilution rates, appreciable sulfur was never reduced to the level of sulfide; therefore full fractionation could not occur.

Fractionation values obtained for sulfide-sulfur were not included in Table XIX for the same reasons as given in the initial chemostat study.

IV. Incorporation of Uniformly ^{14}C-Labelled Yeast Protein Hydrolysate Into Batch Cultures of Isolate #1

The media used in the nutritional studies carried out in continuous culture contained yeast extract. The results of these experiments indicated that elevated yeast extract levels, if lactate was not limiting, stimulated higher cell yields. These experiments did not indicate whether the stimulation was due to the use of yeast extract components for synthesis purposes, for energy purposes (oxidation to CO_2), or was due to a vitamin or cofactor effect.

This experiment was designed to provide information as to the degree of incorporation of yeast protein carbon into the cells of Isolate #1. To determine if significant levels of CO_2 originated from amino acid carbon provided as yeast protein hydrolysate, the design included a trapping mechanism for metabolic CO_2.

Each of the six media used in this experiment (Media #'s 1 to 6 as per Materials and Methods) received 2 x 10^6 dpm of label (as yeast protein hydrolysate). The distribution of ^{14}C in cell fractions and recovered as $^{14}CO_2$ is presented in Table XX. The data indicate that the cultures did not make significant use of the yeast protein material either as a substrate for synthesis or as an energy substrate (based on $^{14}CO_2$ production). The fraction which contained most of the incorporated label never contained more than 7% of the label provided. More total label was taken up when lactate was in excess and yeast extract was limiting (Media #'s 2 and 3). However, as the specific activities of the protein-DNA fractions in these cases were in fact not significantly different from basal levels (Medium #1), it is likely that this was caused by increases in protein production on these media.

TABLE XX. INCORPORATION OF ^{14}C-LABELLED YEAST PROTEIN HYDROLYSATE MATERIAL INTO CELLULAR COMPONENTS OF ISOLATE #1

(2 x 10^6 dpm/culture)

	Medium #					
	1	2	3	4	5	6
Ratios: Lactate	1	3	3	1	3	3
Yeast Extract	1	1	1	3	3	2
Sulfate	1	1	2	2	2	1
$^{14}CO_2$ found in KOH center well (dpm)	875	950	1,050	950	1,100	1,480
Label in total RNA (dpm)	3,972	7,174	6,521	2,895	3,643	4,995
Total RNA/culture (µg)	348	621	522	522	756	711
RNA specific activity (dpm/µg)	11.4	11.5	12.4	5.5	4.8	7.02
Label in total protein-DNA fraction (dpm)	84,120	133,080	122,800	43,040	57,620	77,740
Total protein/culture	8,300	13,800	14,300	11,200	15,200	16,000
Protein specific activity (dpm/µg)	10.1	9.6	8.5	3.8	3.79	4.8
0.3 N $HClO_4$ wash (soluble pool) (dpm)	2,100	4,900	4,200	1,955	1,990	2,490
Culture supernatant	1,965,521	1,778,544	1,735,764	1,811,112	1,847,268	1,844,147
Total label accounted (dpm)	1,965,521	1,924,648	1,870,335	1,859,952	1,911,621	1,930,852
Percentage of label accounted	98.2	96.9	93.5	92.9	95.5	96.5

Where yeast extract (unlabelled) was present in excess (Media #'s 4 and 5), label incorporation in protein-DNA was depressed as compared to levels found in Media #'s 2 and 3. Medium #6 stimulated an intermediate level of ^{14}C incorporation into the protein-DNA fraction. Recoveries of label were in excess of 90% in all media tested.

The overall results are summarized in Table XXI. They suggest that the role of the yeast extract is simply to provide a few necessary amino acids and/or pre-formed vitamins or cofactors. Therefore yeast extract is not required, but is stimulatory to the growth of this Desulfovibrio isolate.

V. Investigations Concerning the Role of Dissimilatory Sulfate Reducers in Crude Oil Catabolism

A review of existing literature concerning sulfate-reducing bacteria and their role in petroleum degradation indicates that indirect evidence such as methylene blue reduction or the formation of black metallic sulfide precipitates was used as evidence of growth of these organisms on petroleum. In many instances, reducing compounds like ascorbic acid, which are readily oxidizable by some bacteria, were included in the medium along with petroleum without having included proper controls. Much of this work also was carried out before advances in analytical chemistry, e.g. gas chromatography, made it possible to readily determine the chemical composition of certain fractions of oil.

Another major problem in interpreting data in the literature is concerned with the difficulties in excluding air from anaerobic environments and the isolation of pure cultures of Desulfovibrio sp. Such problems would permit the growth of aerobes on the petroleum and

TABLE XXI. PERCENT OF THE ^{14}C-LABELLED YEAST PROTEIN HYDROLYSATE INCORPORATED INTO CELLULAR COMPONENTS OR FOUND IN METABOLIC CO_2

Medium #	% as CO_2	% as RNA	% as Protein-DNA	Total
1	0.040	0.996	4.20	5.236
2	0.047	0.358	6.60	7.005
3	0.052	0.326	6.14	6.518
4	0.047	0.144	2.15	2.341
5	0.055	0.182	2.88	3.117
6	0.074	0.249	3.88	4.203

thus allow the sulfate reducers to grow on the products of aerobic oil degradation. Such growth would result in sulfate reduction which would be interpreted as growth of the anaerobes on petroleum.

In an attempt to gain definitive data concerning crude oil degradation by sulfate reducers, the following studies were undertaken:

1. An attempt to show enhancement in numbers of sulfate reducers in soils which had received crude oil applications.
2. To determine whether Isolate #1, derived from petroleum, was in fact capable of utilizing or co-oxidizing crude petroleum under anaerobic conditions.
3. To determine whether Isolate #1 or mixed cultures of sulfate reducers could act as succession populations to aerobic cultures which had already utilized crude petroleum as a sole source of carbon and energy (as per Kuznetsova _et al_, 1965).

A: Incidence of Sulfate Reducing Microorganisms in Areas of Terrestrial Oil Spills

In this study, soil samples were taken, in the spring of 1974, from an oil spill study placed in northern Alberta (Swan Hills) in July, 1972. Oil had been applied to guadruplicate plots at a rate of 60 liters/3x3 meter plot. In an attempt to stimulate the indigenous flora, certain plots had been fertilized (500 lb of elemental nitrogen/acre equivalent and 50 lb of P_2O_5/acre equivalent). As well, certain plots were later limed, limed and fertilized or limed and re-fertilized (377 days after initial fertilizer application).

Table XXII describes the treatments and presents the means of

TABLE XXII. SUMMARY OF SULFATE-REDUCER VIABLE COUNTS (M.P.N.) IN SAMPLES TAKEN FROM OIL SPILL PLOTS IN NORTHERN ALBERTA 665 DAYS AFTER SPILL INITIATION

	Treatment Replicate	Viable Count ($x10^3$)	$\bar{X}(x10^3)$
Control	1	1.09	2.5
	2	2.7	
	3	4.9	
	4	1.7	
Oil	1	49	94
	2	320	
	3	7.9	
	4	1.3	
Oil and Later Liming	1	0.7	2.6
	2	7.0	
	3	1.3	
	4	1.7	
Oil and Later Liming and Fertilizing	1	4.9	46
	2	17	
	3	24	
	4	140	
Oil and Later Fertilizing	1	45	26
	2	32	
	3	22	
	4	7	
Oil and Initial Fertilizing	1	79	40
	2	45	
	3	17	
	4	22	
Oil and Initial Fertilizing and Later Liming	1	49	34
	2	1.3	
	3	16	
	4	70	
Oil and Initial Fertilizing and Later Liming and Fertilizing	1	140	122
	2	49	
	3	170	
	4	130	
Oil and Initial Fertilizing and Later Fertilizing	1	140	124
	2	170	
	3	140	
	4	49	

quadruplicate most probable number counts for sulfate reducers. There was great heterogeneity between replicates; therefore, the data was subjected to T-test analysis. These results, Table XXIII, show only comparisons between test and control plots. A comparison of oil plots with any other treated plots yielded no statistically significant differences. Plots fertilized with nitrogen and phosphate, either at the time of spill initiation or 377 days after, showed high sulfate reducer counts which were statistically significant at the 95% confidence level, from control plots. Plots initially fertilized and then re-fertilized 377 days later, whether or not liming had taken place, also possessed statistically higher sulfate reducer counts both at the 95 and 99% confidence levels. When used as a sole treatment of oiled plots, liming produced a noticeable depression in sulfate reducer counts. This was expected as lime is a commonly used agent in the control of proliferation of sulfate-reducing microorganisms. Counts from plots which had received only oil showed a high degree of variance. Computer projections indicated that approximately seven to eight times the number of samples (28-32) from oil plots would be necessary before these might yield statistically different values from control plot counts. Therefore, if oil with no other treatment, was stimulating sulfate reducer proliferation, it was doing so in a manner which varied between and most likely within plots.

Concomitant studies, (Jobson et al, 1974), revealed that initial fertilizer applications to terrestrial oil spills increased viable counts of aerobic organisms and produced a demonstrable increase in the rate of oil catabolism when compared to the unamended oil plots. It would seem, therefore, that the sulfate reducers could be more

TABLE XXIII. T-TEST OF DATA FROM TABLE XXII, COMPARING TEST PLOT COUNTS TO CONTROL PLOT COUNTS

Treatment	Significance	
	95% Confidence Level	99% Confidence Level
Oil	-	-
Oil and Later Liming	-	-
Oil and Later Liming and Later Fertilizing	-	-
Oil and Later Fertilizing	+	-
Oil and Initial Fertilizing	+	-
Oil and Initial Fertilizing and Later Liming	-	-
Oil and Initial Fertilizing and Later Liming and Later Fertilizing	+	+
Oil and Initial Fertilizing and Later Fertilizing	+	+

under the influence of the applied fertilizer or the products resulting from the temporary increase in numbers of aerobic microorganisms than the effect of merely providing oil as additional carbon substrate. This effect is similar to that already reported by Kuznetsova and co-workers (1965). Their work demonstrated that after an initial increase in aerobic viable counts (using petroleum for substrate), this effect abated to be replaced by a demonstrable increase in sulfate reducer viable counts and H_2S production. It would seem that the field survey work carried out in this thesis might indicate that anaerobic sulfate reducers act as a succession population, using either metabolic end-products or lytic components of the pre-existing aerobic population. In this way, sulfate reducers would be linked with the presence of petroleum, but would only be making indirect use of partially-oxidized petroleum carbon.

B. In Vitro Studies on Petroleum by Isolate #1 and by Mixed Cultures #1 and #2

(i) Test Tube Cultures of Isolate #1 in the Presence of Crude Oil

In order to provide substrate, i.e. oil, in the most reduced section (bottom) of the tubes, North Cantal oil was added adsorbed to silica gel. Non-inoculated controls were treated with three drops of benzene and test samples were inoculated with cells grown in a chemostat on Medium #6. After 55 days incubation at 30°, the oils were recovered by solvent extraction and subjected to chromatographic analyses.

Table XXIV shows that there were some gravimetric changes in the extracted oil when compared with analysis of North Cantal oil taken from storage bottles kept under refrigeration. However, the oil from

TABLE XXIV. RESULTS OF THE TUBE CULTURE OF ISOLATE #1 IN THE PRESENCE OF NORTH CANTAL CRUDE PETROLEUM.

Chromatographic Analysis of Extracted Petroleum

Component	Weight Percent		
	Inoculated	Control	[a]Original
Asphaltenes	17.2	15.2	4.4
Saturates	47.5	43.5	51.8
Aromatics	24.5	27.0	32.2
[b]NSO's	10.9	14.1	12.4
Total Weight Percent	100.0	99.8	100.8

[a] Values correspond to analysis of control oil maintained in storage bottles at 4°.

Length of incubation (30°): 55 days.

[b] NSO: Refers to condensed aromatic ring systems containing nitrogen, sulfur, and oxygen substitutions.

the uninoculated controls showed even more apparent change when compared with the original stored oil. These changes are unavoidable when exposing oils to the culture medium employed in these series of experiments. Long exposure to this medium presumably allows chelation reactions to occur, whereby cations such as iron complex with asphaltene, aromatic, and NSO components thus causing increases in the apparent weight of these fractions (Speers and Whitehead, 1969). Iron and iron salts can be sandwiched between the layers of asphaltene components in much the same way as that observed with colloidal suspensions of clay. The test samples have additional problems with FeS and elemental sulfur contaminating the samples. The elemental sulfur (as rhombic sulfur) originates from re-oxidation of sulfide which occurs during the solvent extraction process. The sulfur behaves chromatographically as an aromatic compound and thus lends weight to this fraction. Some, but not all, of this sulfur can be re-oxidized thiosulfate by reacting it in aqueous conditions with sulfite. Also, it is difficult to quantitatively re-extract the aromatic fraction from this thiosulfate reaction mixture. Problems such as these were not encountered in aerobic culture since iron was not needed for poising purposes and sulfur was not produced during residual oil extractions. The liquid chromatographic data therefore cannot be used for serious comparisons between experiments.

The saturates GLC profiles as illustrated in Figure 18 can be used as evidence for stating as to whether or not n-alkane utilization had occurred. As can be seen in Figure 18, there is virtually no difference between the middle and lower profiles (experimental control and overall control respectively) except that the most abundant n-alkane

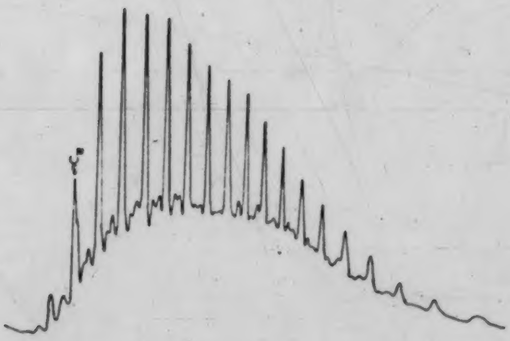
FIGURE 18

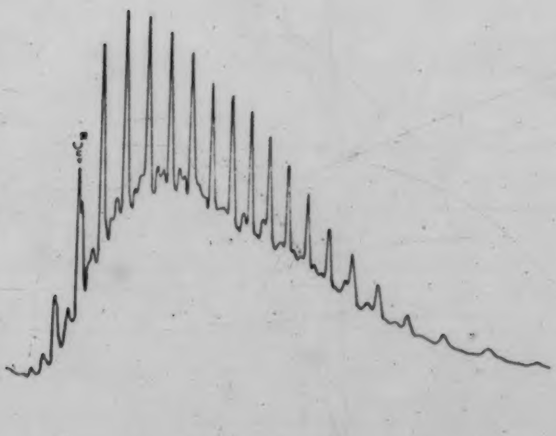
GLC PROFILES OF THE SATURATE
FRACTION FROM RESIDUAL NORTH CANTAL CRUDE
OIL EXTRACTED FROM THE TUBE INCUBATION EXPERIMENT

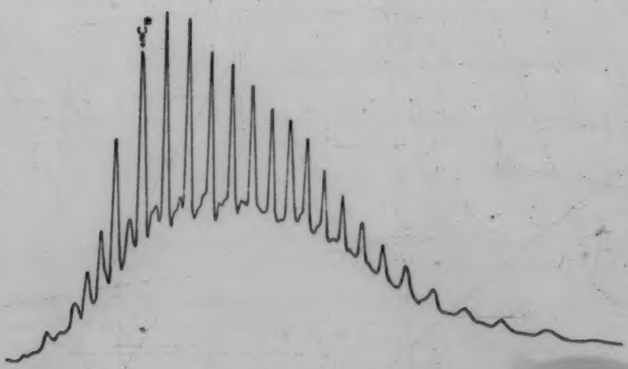
Top: Saturate profile of the crude petroleum extracted from the Isolate #1 tube cultures.

Middle: Saturate profile of the crude petroleum extracted from control incubation tubes.

Bottom: Saturate profile of control North Cantal oil.







peak in the experimental control profile was nC_{20} as opposed to nC_{19} in the overall control. The top profile in Figure 18 shows clearly that incubation of the oil in the presence of the Desulfovibrio isolate did not result in any significant co-oxidation of the n-alkane profile. The cultures, during the incubation period were certainly active, as FeS deposition gradually occurred throughout the complete depth of the silica gel adsorbant. It is clear, however, that their activities during this time were not involved with n-alkane catabolism. In natural oil deposits where degradation has been encountered, it has been the saturate profile which has shown the greatest change resulting in the complete elimination of the n-alkane fraction from the saturate profile. In the experiment described here, however, there was no evidence for such degradation occurring.

(ii) Growth of Isolate #1 and Mixed Cultures MC #1 and MC #2 in the Presence of North Cantal Oil in Sealed, Agitated Flasks

Since the previously-described tube experiment was unsuccessful in demonstrating oil catabolism based on GLC saturate analyses, it was thought that the organism could perhaps utilize petroleum components if more efficient mixing and more efficient mixing and anaerobic conditions were employed. This involved the use of sealed 250 ml flasks, containing 245 ml of medium, which were agitated at 250 rpm on a rotary shaken by lashing the flasks on their sides in conventional shaker clamps. The technique allowed the oil to be continuously in contact with the aqueous medium, thus affording better contact between the substrate and the organism.

Details of the experimental design are presented in Table XXV. The oil was recovered from these flasks after 112 days of incubation at

TABLE XXV. DETAILS OF CULTURING EXPERIMENT USING SEALED, AGITATED FLASKS

Culture Employed	[a]Flask #	Medium Employed	[b]$FeSO_4$ added	[c]Thioglycolate added
Isolate #1	1	#1	+	-
	2	#1	-	+
	3	#8	+	-
	4	#8	-	+
Mixed Sulfate Reducer Population: MC#1	5	#1	+	-
	6	#8	+	-
Mixed Sulfate Reducer Population: MC#2	7	#1	+	-
	8	#8	+	-
Control	9	#1	+	+

a. 0.25 ml of North Cantal oil placed in each flask.

b. $FeSO_4$: 0.5 g (when used)

c. Thioglycolate: 0.25 ml of 10% solution (when used)

NOTE: Control flask received 2 ml of benzene to prevent any growth of cells present in the North Cantal oil.

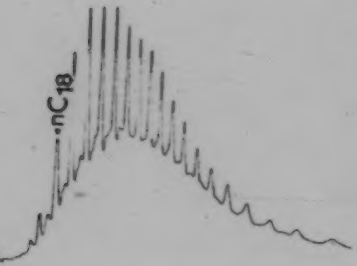
30°, and the results of the liquid chromatographic analyses are summarized in Table XXVI. A composite of the GLC saturate profiles is shown in Figure 19. In nearly every case there was in increase in the percentage of asphaltenes (benzene-soluble and insoluble). In at least three of the analyses, (oils from flask 1: Isolate #1, flask 6: MC #1, and flask 8: MC #2) there had been a reduction in the weight of saturate fractions. However, a comparison of the GLC profiles of the saturate fractions shows that no significant disappearance of n-alkane peaks have occurred. The only noticeable trend seen in the profiles was the occasional apparent buildup of the naphthenic envelope in the mid-portion of the profile (flasks 2, 4, 6, and 7). It is possible that with the mixed cultures employed, sufficient oxygen was initially trapped in the flasks and the aerobic portion of the population utilized some components of the saturate fraction resulting in gravimetric and saturate profile changes. In two cases, (flasks 1 and 8), the saturate profile showed a tiny alteration in the region of nC_{21}-nC_{22}-nC_{23}, possibly indicating some form of initial n-alkane catabolism. It is clear, however, that gravimetric decreases could clearly be related to n-alkane disappearance in aerobic (Jobson, et al, 1972) but not anaerobic systems. Any changes occurring through anaerobic catabolism were taking place via as yet unknown mechanisms which were not revealed by gravimetric or gas chromatographic analyses. Maximum production of hydrogen sulfide corresponded to those flasks containing lactate, yeast extract, and $FeSO_4$, and in all likelihood resulted from normal growth on lactate.

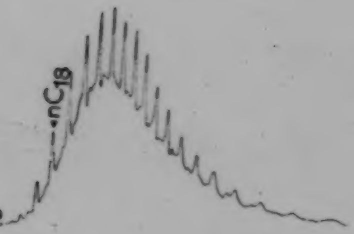
C. Metabolism of Mono-Terminally-Labelled ^{14}C-Octadecane by Isolate #1

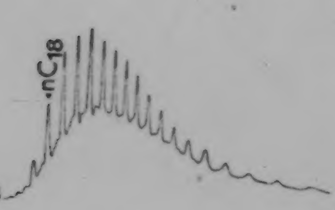
FIGURE 19

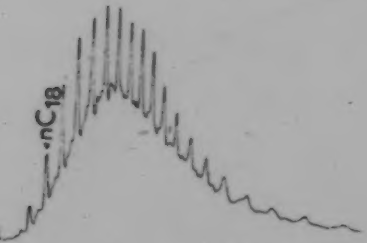
GLC SATURATE PROFILES OF THE RESIDUAL OIL
SAMPLES EXTRACTED FROM THE SEALED SHAKE
FLASKS AFTER 112 DAYS INCUBATION

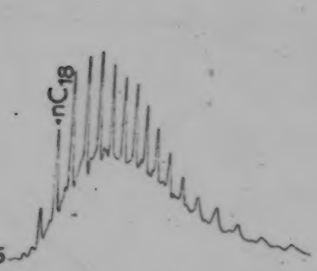
Numbers 1-9 correspond to flask numbers and descriptions as in Tables XXV and XXVI.

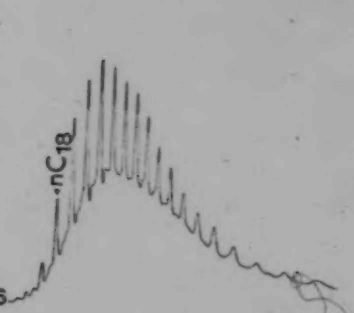

1

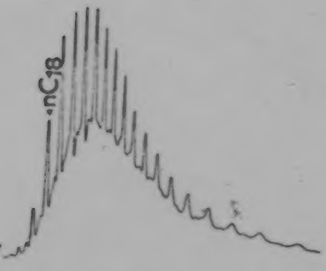

2

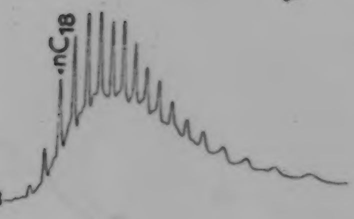

3


4


5


6


7


8

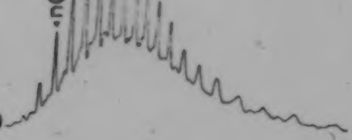

9

TABLE XXVI. SUMMARY OF HYDROGEN SULFIDE PRODUCTION AND LIQUID CHROMATOGRAPHIC ANALYSES OF RESIDUAL OIL EXTRACTED FROM THE SEALED SHAKE FLASK EXPERIMENT

Culture Flask #	H_2S* Production	Weight Percent of Topped Oils			
		Asphaltenes	Saturates	Aromatics	NSO's
1	++++	9.12	39.8	35.5	15.4
2	-	12.1	51.3	28.9	7.60
3	+	12.6	44.9	29.3	13.0
4	-	12.8	49.2	28.3	9.60
5	++++	10.2	42.2	35.8	11.7
6	++	22.4	38.9	45.6	11.9
7	++++	9.17	44.4	32.4	14.0
8	+	8.25	34.4	45.6	11.5
9 (control)	-	11.46	48.0	28.9	11.6
(North Cantal from storage)		4.54	51.8	32.2	12.4

*H_2S production scored from zero to four on the basis of amounts of FeS produced and visually compared with all other flasks. A score of (+) indicates FeS precipitation was barely visible; a score of (++++) indicates very intense production of H_2S which made the flask contents completely black.

The previously-described experiments show that anaerobic catabolism of crude petroleum (particularly the n-alkane portion), if it occurs at all, is an exceedingly slow process. These experiments did not give, however, any indication as to whether carbon from n-alkanes could be assimilated. Therefore Isolate #1 was grown in the presence of ^{14}C-octadecane to see if label could be incorporated into cellular material or respired as CO_2.

The experimental design, employing adapted 250 ml screw-top Erlenmeyer flasks, was as described in Materials and Methods. Isolate #1 was grown on three media (#3, 4 and 7) containing 0.1% thioglycollate and 2.2×10^6 dpm of labelled octadecane per flask. After 10 days incubation at 30°, the KOH was recovered from the center wells and the cells, after centrifugation, were subjected to a Schmidt-Thanhauser fractionation. The final DNA-protein pellets had a high label content (due to adhering octadecane label) which was resolved from this and the RNA fraction by use of thin layer chromatography. There was absolutely no evidence of any label being incorporated into cellular material, nor was any recovered as CO_2. The solvent system used resolved ^{14}C-octadecane from the corresponding acid. There was no indication of any terminal oxidation of octadecane having taken place.

Thus additional evidence had been obtained which, like the previous experiments, indicated that Isolate #1 was unable, under these conditions to utilize octadecane as a carbon or energy source.

Another similar experiment was carried out using ^{14}C-1-labelled stearic acid (2.28×10^6 dpm/culture). In this case Isolate #1 was grown on Media #1, 3, and 4 at 30° for 48 hours. Analyses of the KOH from the centre wells failed to reveal any significant amounts of $^{14}CO_2$

production. As well, none of the fractionated cell components revealed any label incorporation. Thus oxidation of octadecane to stearic acid would not, in all likelihood, allow Isolate #1 to utilize the substrate for carbon or energy.

D. Demonstration of Metabolism or Growth of Desulfovibrio sp. in Association with Aerobic Microbial Catabolism of Crude Petroleum

(i) Metabolism of Isolate #1 in Aerobic Mixed Cultures Growing on North Cantal Oil

Attempts to detect changes in crude petroleum using either Isolate #1 or mixed sulfate-reducing cultures under enforced anaerobic conditions were quite unsuccessful. Nevertheless, the fact remains that viable sulfate-reducing microorganisms can be found in association with crude petroleum in natural deposits which show degradation attributed to microbiological processes. The work of Kuznetsova (1965) gave clear evidence of a microbial succession with initial growth by aerobic organisms such as Pseudomonas sp. followed later by an increase in sulfate reducer activity. The oil spill plot survey described earlier did not clearly demonstrate succession as samples were not taken with respect to time. For this reason, in vitro lab experiments were set up to try and duplicate a succession series as one might find it in nature. If, as Kuznetsova's work indicated, aerobic organisms preceeded anaerobic growth, aerobes may well have been the agents of initial hydrocarbon oxidation, thus providing substrate for the later anaerobes. Therefore an experiment was designed to test the ability of Isolate #1 to grow or metabolize in cultures of aerobes using crude oil as a sole carbon substrate.

The aerobic culture used in this experiment was one enriched three years previously on North Cantal crude petroleum. The culture had been transferred bi-weekly to fresh inorganic salts medium with 0.1% North Cantal oil as the sole carbon source. Thus contamination of the culture with non-hydrocarbon compounds was very unlikely. This culture could achieve almost complete degradation of the n-alkane profile (excluding pristane and phytane) within a fourteen day period, Jobson et al (1972). This mixed aerobic population could not grow under anaerobic conditions using crude oil as a sole carbon source, nor would it produce any detectable H_2S under such conditions. Two cultures of this mixed population were transferred four times on Medium #8 + 0.1% North Cantal oil to ensure that nitrate, present in their normal maintenance medium, was decreased in concentration so as not to interfere with the growth of sulfate reducers. The fourth set of transfers were allowed to incubate for 14 days at 30° and treated in the following manner. One culture was first centrifuged to remove the bulk of the cells and then filter-sterilized using 0.45 μm Millipore filters to remove any remaining intact cellular material. The filtrate was used to fill a 500 ml bottle containing one gram of iron wool for poising purposes. A second culture bottle was filled with intact aerobic culture containing aerobic cells, residual crude oil, and spent medium. This bottle was also poised with iron wool. A third culture bottle was filled with fresh Medium #8 and 0.5 ml of fresh North Cantal petroleum and also poised with iron wool. Each bottle was then inoculated with 5.0 ml of Isolate #1 culture by pipetting the cells into the region of the iron wool. These three bottles were incubated at 30°. Plate III is a photograph of the three bottles 72 hours after inoculation.

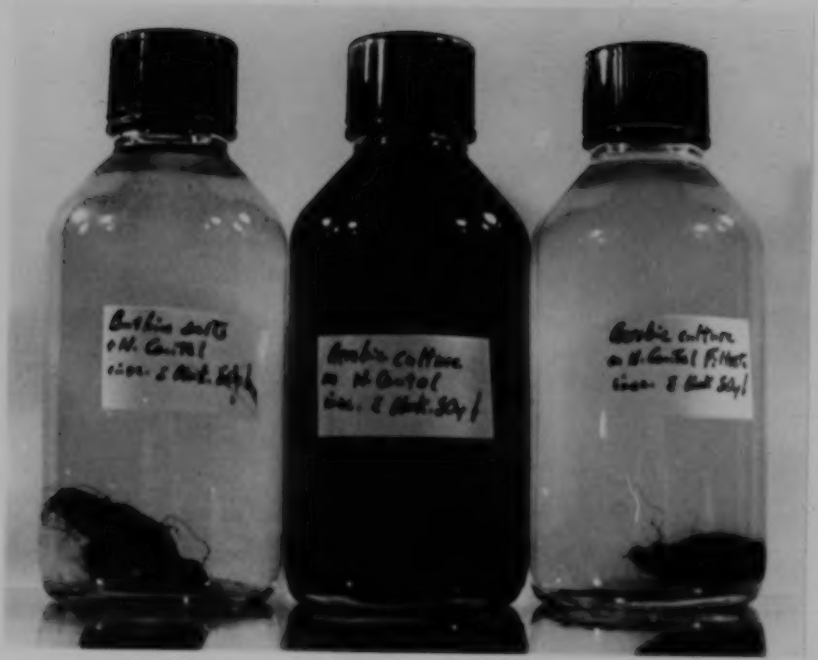
PLATE III

METABOLISM BY ISOLATE #1 ON SPENT, AEROBIC OIL-UTILIZING CULTURE

Left: Medium #8 + 0.5 ml fresh North Cantal oil 72 hours after inoculation with Isolate #1.

Middle: Fourteen day old aerobic culture in Medium #8 + 0.1% (vol/vol) North Cantal oil 72 hours after inoculation with Isolate #1. (positive H_2S production).

Right: Filtrate from 14 day old aerobic culture in Medium #8 + 0.1% (vol/vol) North Cantal oil 72 hours after inoculation with Isolate #1.


Aerobic culture
on N. Control
inoc. 8 Aut. 50g/l
Aerobic culture
on N. Control F. Nat.
inoc. 8 Aut. 50g/l

The bottle to the left of the photograph, Medium #8 + fresh North Cantal oil, shows no H_2S production and thus no apparent growth or metabolism of Isolate #1. The bottle on the right-hand side of the photograph, filtered aerobic culture, also failed to show any visible evidence of sulfate reduction. Only the middle bottle, containing intact 14 day old culture, showed significant sulfate reduction indicating metabolism and/or growth of Isolate #1. Unfortunately, attempts to carry out viable count estimations of these bottles failed since, in the case of the positive bottle, facultative organisms were still high in number and caused overgrowth of the Brewer's agar; thus only a few black colonies were seen in the 10^{-6} dilution agar tubes. No positive tubes were seen in the counts carried out on the other two bottles. Thus Plate III demonstrates that sulfate reducer metabolism, resulting in H_2S production, was occurring in the middle intact culture bottle; but suggestions of actual growth of Isolate #1 are speculative.

Analyses of the oils were not undertaken, because after 14 days of aerobic incubation, the oil had already been severely degraded before the culture was used as test medium for the anaerobic experiment. One can be quite certain that the substrates for metabolism in the presence of whole aerobic culture were hydrocarbon-derived, since this was the only initial source of carbon and energy. The lack of metabolism in the filtered culture supernatant could indicate that the substrates utilized by Isolate #1 could be associated with the intact aerobic bacterial cell surfaces or that co-participation of the actual aerobic culture, in some form, was necessary for the stimulation of Isolate #1 metabolism.

(ii) Enrichment of a Mixed Culture of Dissimilatory Sulfate Reducing Microorganisms from Norman Wells Refinery Soil

The successful demonstration of the growth or metabolism of Isolate #1 in reduced, spent, cultures of a mixed aerobic population grown on crude oil as its sole carbon source suggested the carrying out of an experiment to see if both an aerobic and anaerobic population could be enriched from the same soil sample.

An oil-soaked soil sample obtained from the Norman Wells refinery site in 1974, and known to contain an active sulfate-reducing population, was used as source of inoculum for aerobic enrichment (Jobson, 1974) and North Cantal petroleum as sole carbon source. After the 7th day of the 4th transfer, the culture was used to fill a 500 ml bottle to which was added a one gram portion of iron wool and a one gram portion of the refinery soil. The contents of the bottle turned completely black after nine days incubation at 30° indicating sulfate reduction had taken place.

Phase contrast microscopy revealed an abundance of vibrio-shaped organisms, some spore-forming rods, and many rod-shaped organisms. The first type looked like a Desulfovibrio sp., and the presence of spore-containing rods suggested the possible presence of Desulfotomaculum species. This black, anaerobic mixed culture was used as inoculum for the second phase of the experiment where three more bottles were set up. The first was filled with what was now transfer 6 of the initial aerobic enrichment, plus a one gram portion of iron wool. A second bottle was provided with a one centimeter layer of silica gel to which was added 0.5 ml of North Cantal oil, one gram of iron wool and 500 ml of Medium #8. Finally sterile, dry Na_2SO_3 was added to 0.1% (w/v). It was thought

PLATE IV

PHOTOGRAPH OF BOTTLES AFTER TWENTY DAYS INCUBATION AT 30° DEMONSTRATING H_2S PRODUCTION

Left: Medium #8 (+ 0.1% Na_2SO_3) + North Cantal Oil-impregnated silica gel inoculated with 10 ml of initial anaerobic enrichment culture.

Middle: Spent aerobic culture growing on North Cantal Petroleum inoculated with 10 ml of initial anaerobic enrichment culture.

Right: Medium #8 + North Cantal-impregnated silica gel inoculated with 10 ml of initial anaerobic enrichment culture.

NW enrichment inoc. into Butlins salts + CaCO3 + Na2SO3 (0.02% ...) + N. Control (0.5ml) 13/8/74
NW enrichment inoc. into aerobic N. Control culture + Fe Wool 13/8/74
NW enrichment inoc. into Butlins salts + CaCO3 + 0.5ml N.C. cell. 13/8/74

that the presence of a primary electron acceptor not requiring activation might aid the growth of the sulfate reducing component of the population. A third bottle was set up like the second except that no Na_2SO_3 was added. Each of the three bottles was then inoculated with 10 ml of the initial anaerobic culture. Between the ninth and tenth day of incubation at 30°, the bottle of re-inoculated aerobic culture again turned very black while the other two developed only slight evidence of H_2S production even after a further incubation period of ten days. Plate IV is a photograph of the bottles 20 days after initial incoulation. It is clear that the middle bottle, containing inoculated spent aerobic culture, is the only one of the three which shows significant H_2S production, indicating at least active metabolism of sulfate reducers. Viable count estimations for sulfate-reducers using Brewers agar in tubes was unsuccessful because most of the agar suffered severe cracking and spreading due to gas production by components of the population.

This experiment would again suggest that sulfate reducing microorganisms can make indirect use of petroleum hydrocarbons by utilizing materials from aerobic populations which utilized petroleum as their sole carbon and energy source. As well, both the aerobic and the anaerobic populations were successfully enriched from the same soil sample.

Experiments like these indicate how one might implicate sulfate reducing microorganisms as agents of oil catabolism in natural deposits. If, as Kuznetsova's work (1965) indicates, the initial aerobic action on petroleum is, on a time scale, significantly removed from the period of intense sulfate reduction and sulfate-reducer growth, the aerobic components of the population may have disappeared long before the presence of sulfate-reducers is realized. Thus, if one studied the overall population at the time of maximal H_2S production and carried out analyses of oil at this time, an apparent link between sulfate-reducer growth and oil catabolism would be an obvious conclusion. This of course would be wrong in that catabolism of the oil would have already occurred. I believe this sequence of events has done much to lend confusion to the whole concept of "anaerobic" degradation of crude petroleum.

(iii) Evaluation of the Medium used by ZoBell to Demonstrate Crude Oil Catabolism

The work of ZoBell (1947) was consulted several times during the period of time that personal attempts to demonstrate direct utilization of crude oil in an anaerobic environment by sulfate reducers was consistantly yielding negative results. The two-part paper described work by ZoBell concerning the release of crude petroleum from Athabasca Tar sand or from shale. In the course of his discussions, he frequently referred to his apparent successes in showing that both pure and mixed sulfate reducing cultures would grow on petroleum hydrocarbons. The composition of the basic medium he employed in these studies is as listed in Materials and Methods. He stated in his papers that one could substitute other substrates such as crude petroleum in place of lactate and achieve successful growth of sulfate reducers.

Except for the use of sea water, this medium was duplicated in our laboratory and used for an enrichment study. In the initial experiment, a one centimeter layer of oil-soaked silica gel was placed in a 250 ml screw-top Erlenmeyer and filled with ZoBell's medium. A one gram portion of iron wool and a one gram quantity of the soil taken from the

Norman Wells oil refinery was added to the flask which was sealed and incubated at 30°. Within four days, the flask contents were completely blackened, indicative of significant H_2S production. However, there was no way of knowing whether the organisms were utilizing the crude oil, nutrients from the soil inoculum, or the ascorbate (used for poising purposes by ZoBell). Therefore, three more flasks were set up, one containing ZoBell's medium, ascorbate, iron wool and oil; the second contained ZoBell's medium, ascorbate, iron wool but no oil; and the last flask contained ZoBell's medium, iron wool, oil but no ascorbate. The three flasks were each inoculated with 5 ml of original enrichment. After three days incubation at 30°, the flasks containing ascorbate showed significant H_2S production; while the flask lacking ascorbate but given iron for poising showed no evidence of H_2S production. These results remained constant through twenty additional days of incubation. A photograph of the three flasks appears in Plate V.

These results indicate that the ascorbate in the medium was apparently the substrate used for metabolism. Careful study of ZoBell's paper failed to reveal whether or not ascorbate was checked as a possible substrate. This coupled with the lack of chemical data showing oil degradation casts doubt concerning the validity of ZoBell's conclusions with respect to _Desulfovibrio_ sp. using crude petroleum as a direct growth substrate. The paper of MacPherson and Miller (1963) cautions against the use of ascorbate as a poising agent when checking compounds as possible substrates for the growth of _Desulfovibrio_ sp.

PLATE V

PHOTOGRAPH OF GROWTH FLASKS AFTER 72 HOURS INCUBATION AT 30°.USING ZOBELL'S GROWTH MEDIUM

Left: Flask contains ZoBell's Salts Medium plus ascorbate with 0.25 ml North Cantal Oil impregnated in silica gel.

Middle: Flask contains ZoBell's Salts plus ascorbate with no added crude oil.

Right: Flask contains ZoBell's Salts minus ascorbate with 0.25 ml North Cantal Oil impregnated in silica gel.

CONCLUSIONS

The sulfate-reducer enriched from North Cantal crude petroleum and used in the majority of experiments in this thesis has been provisionally identified as being an example of Desulfovibrio vulgaris, var oxamicus. Like many other species of Desulfovibrio it uses only a few, simple organic substrates such as lactate, n-propanol, ethanol, oxaloacetate, and pyruvate, for electrons and carbon. It does not require any pre-formed amino acids or vitamins, but is stimulated if yeast extract is present, provided of course that sufficient lactate and sulfate are also present. The labelled yeast protein hydrolysate uptake study indicates that very little carbon from yeast extract is either incorporated or metabolized to CO_2. Because of the ability of this organism to use simple organic compounds as sources of carbon and energy, it is easy to visualize situations where it could successfully grow on the end-products of aerobic fermentations.

Results of both batch and continuous culturing of this organism indicate that growth is best measured by monitoring protein, RNA, and DNA levels rather than viable counts. It would appear that the decision to study this isolate under conditions of continuous culture was wise in that more details concerning protein, RNA, and DNA metabolism could be elucidated. For instance, as a result of the use of continuous culture, it is apparent that Isolate #1 either maintains large levels of DNA, or its RNA suffers rapid turnover during the course of growth. Good control of RNA and protein metabolism is evident under the varying chemostat conditions employed; however, DNA metabolism appears to be under loose control both at very slow and at very fast growth rates.

Also as a result of using continuous culture techniques, it appears that Isolate #1 does not produce accumulating amounts of sulfite beyond intermediate growth rates. Thiosulfate, ~~on the other~~ hand, accumulates at an ever-increasing rate as growth rates are increased. This is taken as good initial evidence that, in this organism, thiosulfate reduction can potentially be the rate limiting step of dissimilatory sulfate reduction.

In terms of the media employed in these continuous culture studies, it appears that Isolate #1 grows most intensively at a growth rate no higher than 0.08 hr^{-1}. However, maximum hourly production levels are achieved at growth rates in the order of 0.26 hr^{-1}.

Maximum sulfur isotope fractionation, although never excessively high with Isolate #1, was not only a function of growth rate, but of the combined levels of electron donor (lactate), electron acceptor (sulfate), and of the level of yeast extract provided.

Direct and significant catabolism of crude petroleum by Isolate #1 or MC #1 and #2, under anaerobic conditions, cannot be demonstrated, nor is there any evidence of labelled octadecane uptake or metabolism. This indicates that such reactions either do not occur, or do so at rates imperceptable in terms of the incubation times used.

Attempts to relate heightened levels of sulfate-reducer activity with the presence of oil spills are apparently very difficult when these spills have received exogenous nitrogen and phosphorous.

The conclusion of the study carried out for this thesis is that one cannot tell whether stimulation of the sulfate-reducers is due to the enhanced nutritional status or the presence of higher numbers of aerobic organisms in the fertilized oil spill.

There is good initial evidence that both Isolate #1 and enriched mixed cultures of sulfate-reducers can respire and perhaps divide in spent aerobic cultures which have used crude petroleum as a sole source of carbon and energy. Moreover, both the aerobic, and the sulfate reducing populations can be sequentially enriched from the same soil indicating that succession by sulfate-reducers could be possible in almost any environment where crude petroleum might be spilled.

In all likelihood, the significant role of sulfate-reducers, in terms of petroleum catabolism, hinges around their abilities to act as succession populations. In doing so, they probably make use of metabolic end-products resulting from classical utilization of crude petroleum by aerobic microbial populations. This suggestion is not incompatible with actual in situ data, which indicates that in oil deposits showing apparent microbial degradation, significant levels of dissolved oxygen can be found in ground waters. It is therefore probable that utilization of petroleum carbon by sulfate-reducers in an anaerobic environment is an indirect process dependent upon initial aerobic oxidations by separate microbial populations.

BIBLIOGRAPHY

Akagi, J.M., and V. Adams. 1966. Oxidation of pyruvic acid by cell-free extracts of *Desulfovibrio desulfuricans*. Bact. Proc.: 215.

Ashwell, G. 1957. Colorimetric analysis of sugars. Methods in Enzymology, III: 73-107.

Baars, J.K. 1930. Over Sulfaatreductie door Bacterien. Dissertation, W.D. Meriema, N.V., Delft. (as described by Butlin, 1949).

Bailey, N.J.L., A.M. Jobson, and M.A. Rogers. 1973. Bacterial degradation of crude oil: Comparison of field and experimental data. Chem. Geol. 11: 203-221.

Barten, L.L., Jean LeGall, and Harry D. Peck, Jr. 1972. Oxidative phosphorylation in the obligate anaerobe *Desulfovibrio gigas*. Proceedings of a Symposium held at Bloomington Indiana, Oct. 12-15. Ed. by Anthony San Pietra and Howard Gest. Academic Press, New York.

Bauchop, T., and S.R. Elsden. 1960. The growth of microorganisms in relation to their energy supply. J. Gen. Microbiol. 23: 457-469.

Beck, J.V. 1947. Penn grade progress on use of bacteria for releasing oil from sands. Producers Monthly 11: 13-19.

Beijerinck, M.W. 1895. Uber spirillum desulfuricans als Ursache von sulfat-reduction. Zentr. Bakteriol., Parasitenk., Abt II, 1: 1-9 (as described in Petroleum Microbiology, Davis, 1967).

Bertolacini, R.J., and J.E. Barney, II. 1957. Colorimetric determination of sulfate with barium chloranilate. Anal. Chem. 29, 2: 281-283.

Blasius, E., G. Horn, A. Knochel, J. Munch, and H. Wagner. 1968. In *Inorganic Sulfur Chemistry*, 1st Edition, p. 201-231. Ed. by G. Nickless. Elsevier Publishing Company, N.Y.

Booth, G.H. 1964. Sulfur bacteria in relation to corrosion. J. Appl. Bacteriol. 27: 174-181.

Bowden, T.J., F.C. Happold, and B.F. Taylor. 1966. Studies on adenosine-5'-phosphosulfate reductase from *Thiobacillus denitrificans*. Biochim. Biophys. Acta 118: 566-576.

Breed, R.S., E.G.D. Murray, and N.R. Smith. 1957. In Bergey's Manual of Determinative Bacteriology, 7th Edition. Williams and Wilkins, Baltimore, 1094 pp.

Bryant, M.P. 1969. Symbiotic association of certain ethanol and lactate fermenting bacteria. Abstracts of the 158th Meeting of the American Chemical Society, Microbiology Section, p. 18.

Bryant, M.P., E.A. Wolin; M.J. Wolin, and R.S. Wolfe. 1967. Methano-bacillus omelianskii, a symbiotic association of two species of bacteria. Archiv fur Mikrobiol. 59: 20-31.

Buchanan, R.E., and N.E. Gibbons. 1974. In Bergey's Manual of Determinative Bacteriology, 8th Edition. Williams and Wilkins, Baltimore, 1246 pp.

Bunker, H.J. 1939. Factors influencing the growth of Vibrio desulfuricans. Intern. Congr. Microbiol., 3rd., New York: p. 64.

Butlin, K.R., M.E. Adams, and M. Thomas. 1949. The isolation and cultivation of sulfate reducing bacteria. J. Gen. Microbiol. 3: 46-59.

Butlin, K.R., M.E. Adams, and M. Thomas. 1949. Sulfate reducing bacteria and internal corrosion of ferrous pipes conveying water. Nature 163: 26-27.

Campbell, L.L., and J.R. Postgate. 1965. Classification of the spore-forming sulfate reducing bacteria. Bacteriol. Rev. 3: 359-363.

Campbell, N.E.R., and H. Lees. 1967. In Soil Biochemistry, vol. 1, 1st Edition, p. 197-214. Ed. by A.D. McLaren, and G.H. Peterson. Marcel Dekker, Inc., New York.

Davis, J.B. 1967. In Petroleum Microbiology, 1st Edition, p. 105. Elsevier Publishing Company, Amsterdam, London and New York.

Dawson, P.S.S. 1963. A continuous-flow culture apparatus (the cyclone column unit). Can. Jour. Microbiol. 9: 671-687.

Decker, K., K. Jungermann, and R.K. Thauer. 1970. Energy production in anaerobic organisms. Angew Chem. internat. Edit. 9, 2: 138-158.

Delden, A. van. 1903. Beitrag zur Kenntnis der Sulfat-Reduktion durch Bakterien. Zbl Babt. 11, 31: 113. (as described by Butlin, 1949).

DeLey, J. 1970. Reexamination of the association between melting point, buoyant density, and chemical base composition of Deoxyribonucleic acid. J. Bacteriol. 101 3: 738-754.

Dostalek, M. and A. Rosypalova. 1957. The action of microorganisms on petroleum hydrocarbons. Cesk. Mikrobiol. 2: 43-47. (as described by Davis, 1967).

Elliott, W.H. 1959. Amino-acetone: its isolation and role in metabolism. Nature 183: 1051-1052.

Ellman, G.L., K.D. Courtney, V. Andres, Jr., and R.M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7: 88-95.

Ettre, L.S., and F.J. Kabot. 1963. Relative response of fatty acid methyl esters on a flame ionization detector. J. Chromatog. 11: 114-116.

Grossman, J.P., and J.R. Postgate. 1953. Cultivation of sulfate-reducing bacteria. Nature 171: 600-601.

Hammar, H.E. 1934. In Problems of Petroleum Geology, p. 35. Am. Assoc. Petrol. Geologists. Tulsa, Okla.
(from Davis, 1967).

Harrison, A.G., and H.G. Thode. 1958. Mechanism of the bacterial reduction of sulfate from isotope fractionation studies. Trans. Faraday Soc. 54: 84-92.

Harrison, A.G., and H.G. Thode. 1957. The kinetic isotope effect in the chemical reduction of sulfate. Trans. Faraday Soc. 53: 1648-1651.

Hatchikian, E.C. and J. LeGall. 1972. Evidence for the presence of a b-type cytochrome in the sulfate reducing bacterium, Desulfovibrio gigas and its role in the reduction of fumarate by molecular hydrogen. Biochem. Biophys. Acta 267: 479-484.

Herbert, D., P.J. Phipps, and R.E. Strange. 1971. In Methods in Microbiology, vol. 5B, p. 250. Ed. by J.R. Norris and D.W. Ribbons. Academic Press, New York.

Ishimoto, M. 1959. Sulfate reduction in cell-free extracts of Desulfovibrio. J. Biochem., Tokyo 41: 763-770.

Ishimoto, M., J. Koyama, and Y. Nagai. 1954. Biochemical studies on sulfate reducing bacteria. IV. The cytochrome system of sulfate reducing bacteria. J. Biochem., Tokyo 41: 763-770.

Jones, H.E. 1972. Cytochromes and other pigments of dissimilatory sulfate reducing bacteria. Archiv. Mikrobiol. 84: 207-224.

Jones, H.E. 1971. A re-examination of Desulfovibrio africanus. Arch. Mikrobiol. 80: 78-86.

Jones, G.E., and R.L. Starkey. 1957. Fractionation of stable isotopes of sulfur by microorganisms and their role in deposition of native sulfur. Appl. Microbiol. 5: 111-118.

Jobson, A., M. McLaughlin, F.D. Cook, and D.W.S. Westlake. 1974. Effect of ammendments on the microbial utilization of oil applied to soil. Appl. Microbiol. 27, 1: 166-171.

Jobson, A.M., F.D. Cook, and D.W.S. Westlake. 1972. Microbial utilization of crude oil. Appl. Microbiol., 23: 1082-1089.

Jobson, A.M. 1971. Bacterial metabolism of crude petroleum. MSc. thesis, 1971. University of Alberta, Edmonton.

Kaplin, I.R. and S.C. Rittenberg. 1964. Microbiological fractionation of sulfur isotopes. J. Gen. Microbiol. 34: 195-212.

Kaplin, I.R., T.A. Rafter, and J.R. Halston. 1960. Sulfur isotopic variations in nature. Part 8 - Application to some biochemical problems. New Zealand Journal of Science: 338-361.

Kemp, A.L.W., and H.G. Thode. 1968. The mechanism of bacterial reduction of sulfate and of sulfite from isotope fractionation studies. Geochim. Cosmochim. Acta 32: 71-91.

Kidman, A.D., R. Yanagihara, and R.N. Asato. 1969. Comparative studies of bacterial hydrogenases. Biochim. biophys. Acta 191: 170-173.

Kjeldgaard, N.O. 1967. In Advances in Microbial Physiology, vol. 1, p. 39-95. Ed. by A.H. Rose and J.F. Wilkinson. Academic Press, London.

Kobayashi, K., S. Tachibana, and M. Ishimoto. 1969. Intermediary formation of trithionate in sulfite reduction by a sulfate reducing bacterium. J. Biochem., Tokyo 65: 155-157.

Krouse, H.R., R.G.L. McCready, S.A. Husain, and J.N. Campbell. 1967. Sulfur isotope fractionation by Salmonella species. Can. J. Microbiol. 13: 21-25.

Kuhr, C.A., H. von Wolzogen, and I.S. van der Vlugt. 1934. The graphitization of cast iron as an electrochemical process in anaerobic soils. Water 18: 147. (as described by Butlin, 1949).

Kuznetsova, V.A., and V.M. Gorlenko. 1965. Effect of temperature on the development of microorganisms from flooded strata of the Romashkino oil field. Mikrobiologiya 34: 274-278.

Lee, J.P., J. LeGall, and H.D. Peck, Jr. 1973. Isolation of assimilatory and dissimilatory type sulfite reductases from Desulfovibrio vulgaris. J. Bacteriol. 15, 2: 529-542.

Lee, J.P., J. LeGall, and H.D. Peck, Jr. 1971. Purification of an assimilatory-type of sulfate reductase from Desulfovibrio vulgaris. Federation Proceedings 30: 1202.

LeGall, J., and J.R. Postgate. 1973. The physiology of sulfate reducing bacteria. Adv. Microb. Physiol. 10: 81-125.

LeGall, J. 1968. Purification partielle et etude de la NAD: rubredoxine oxydoreductase de D. gigas. Annls. Inst. Pasteur, Paris 114: 109-115. (as described by LeGall and Postgate, 1973).

LeGall, J. and E.C. Hatchikian. 1967. Purification et proprietes d'une flavoproteine intervenant dans la reduction du sulfite. C.r. hebd. Seanc. Acad. Sci., Paris 264: 2580-2583. (as described by LeGall and Postgate, 1973).

LeGall, J. and N. Dragoni. 1966. Dependence of sulfite reduction on a crystallized ferredoxin from Desulfovibrio gigas. Biochem. Biophys. Res. Comm. 23: 145-149.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall, 1951. Protein measurement with the folin plenol reagent. J. Biol. Chem. 193: 265-275.

MacPherson, R., and J.D.A. Miller. 1963. Nutritional studies on Desulfovibrio desulfuricans using chemically-defined media. J. Gen. Microbiol. 31: 365-373.

Man, M. and R.G. Bryant. 1974. Reactions of thiosulfate and sulfite ions with DTNB: interference in sulfhydral group analysis. Anal. Biochem. 57: 429-431.

Marmur, J., S. Falkow, and M. Mandel. 1963. New approaches to bacterial taxonomy. Ann. Rev. Microbiol. 17: 329-372.

Marmur, J. and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. 5: 109-118.

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3: 208-218.

Mechalas, B.J., and S.C. Rittenberg. 1960. Energy coupling in Desulfovibrio desulfuricans. J. Bacteriol. 80: 501-507.

Meinschien, W.G. 1969. In Organic Geochemistry, Methods and Results, 1st Edition, p. 330. Ed. by G. Eglinton and M.T.J. Murphy. Springer-Verlag, New York.

Miller, J.D.A. and D.S. Wakerley. 1966. Growth of sulfate-reducing bacteria by fumarate dismutation. J. Gen. Microbiol. 43: 101-107.

Nakai, N., and M.L. Jensen. 1964. The kinetic isotope effect in the bacterial reduction and oxidation of sulfur. Geochim. Cosmochim. Acta 28: 1893-1912.

Neish, A.C. 1952. In Analytical Methods for Bacterial Fermentations. 2nd Edition. p. 42. N.R.C. No. 2952.

Pankhurst, E.S. 1971. In The Society for Applied Bacteriology Technical Series #5: Isolation of Anaerobes, 1st Edition, p. 228. Ed. by D.A. Shapton and R.G. Board. Academic Press, London.

Peck, H.D., Jr. 1974. The evolutionary significance of inorganic sulfur metabolism. Twenty-fourth symposium of the Society for General Microbiology held at Imperial College, London, April 1974. Cambridge University Press.

Peck, H.D., Jr. 1966. Phosphorylation coupled with electron transfer in extracts of the sulfate reducing bacterium Desulfovibrio gigas. Biochem. Biophys. Res. Comm. 22: 112-118.

Peck, H.D., Jr., Deacon T.E., and J.T. Davidson. 1965. Studies on adenosine 5'-phosphosulfate reductase from Desulfovibrio desulfuricans and Thiobacillus thioparus. I. The assay and purification. Biochem. Biophys. Acta 96: 429-446.

Peck, H.D., Jr. 1959. The ATP-dependent reduction of sulfate with hydrogen in extracts of Desulfovibrio desulfuricans. Proc. Natl. Acad. Sci. (U.S.) 45: 701-708.

Postgate, J.R., and L.L. Campbell. 1966. Classification of Desulfovibrio species, the non-sporulating sulfate reducing bacteria. Bacteriol. Rev. 30, 4: 732-738.

Postgate, J.R. 1965. Recent advances in the study of the sulfate reducing bacteria. Bacteriol. Rev. 20: 425-441.

Postgate, J.R. 1960. On the autotrophy of Desulfovibrio desulfuricans. Z. Allgem. Mikrobiol. 1: 53-56. (as described by LeGall and Postgate, 1973).

Postgate, J.R. 1960. The economic activities of sulfate reducing bacteria. Progr. Ind. Microbiol. 2: 49-69.

Postgate, J.R. 1959. Sulfate reduction by bacteria. Ann. Rev. Microbiol. 13: 505-520.

Postgate, J.R. 1956. Cytochrome C$_3$ and desulfoviridin; pigments of the anaerobe Desulfovibrio desulfuricans. J. Gen. Microbiol. 14: 545-572.

Postgate, J.R. 1953. On the nutrition of Desulfovibrio desulfuricans: a correction. J. Gen. Microbiol. 9: 440.

Postgate, J.R. 1951. On the nutrition of Desulfovibrio desulfuricans. J. Gen. Microbiol. 5: 714-724.

Rees, C.E. 1973. A steady-state model for sulfur isotope fractionation in bacterial reduction processes. Geochim. Cosmochim. Acta 37: 1141-1162.

Robbins, P.W., and F. Lipmann. 1958. Separation of the two enzymatic phases in active sulfate synthesis. J. Biol. Chem. 233: 681-685.

Rosenfeld, W.D. 1960. Petroleum prospecting method. U.S. Pat.: 2,921,003.

Rosenfeld, W.D. 1947. Anaerobic oxidation of hydrocarbons by sulfate reducing bacteria. J. Bacteriol. 54: 664-665.

Roy, A.B., and P.A. Trudinger. 1970. In The Biochemistry of Inorganic Compounds of Sulfur. 1st Edition. p. 326. London: Cambridge University Press.

Saunders, G.F., L.L. Campbell, and J.R. Postgate. 1964. Base composition of deoxyribonucleic acid of sulfate reducing bacteria deduced from buoyant density measurements in cesium chloride. J. Bacteriol. 87: 1073-1078.

Schildkraut, C.L., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. 4: 430-443.

Schmidt, G., and S.J. Thannhauser. 1945. A method for the determination of deoxyribonucleic acid, Ribonucleic acid, and phosphoproteins in animal tissues. J. Biol. Chem. 161: 83-89.

Senex, J.C. 1962. Some considerations on the energetics of bacterial growth. Bacteriol. Rev. 26: 95-107.

Shturm, L.D. 1951. Role of the sulfate reducing bacteria in the life and history of petroleum deposits. I.M. Gubkina Akad. Nauk. S.S.S.R.: 275-286.
(as described by Davis, 1967).

Sigal, N., J.C. Senez, J. LeGall, and M. Sebald. 1963. Base composition of the deoxyribonucleic acid of sulfate reducing bacteria. J. Bacteriol. 85, 6: 1315-1318.

Sorokin, Y.I. 1966. Role of carbon dioxide and acetate in biosynthesis by sulfate reducers. Nature 210: 551-552.

Sorokin, Y.I. 1961. Utilization of CO_2 in biosynthesis by microorganisms. Proc. Intern. Congr. Biochem., 5th Moscow, 22: 453.

Speers, G.C., and E.V. Whitehead. 1969. In Organic Geochemistry, 1st Edition, pp. 638-667. Ed. by G. Eglinton and M.T.J. Murphy. Springer Verlag, N.Y.

Stouthamer, A.H., and Corry Bettenhoussen. 1973. Utilization of energy for growth and maintenance in continuous culture and batch cultures of microorganisms: a reevaluation of the method for the determination of ATP production by measuring molar growth yields. Biochim. Biophys. Acta 301: 53-70.

Suh, B., and J.M. Akagi. 1969. Formation of thiosulfate from sulfite by *Desulfovibrio vulgaris*. J. Bacteriol. *99*: 210-215.

Szybalski, W., and E.H. Szybalski. 1971. *In Procedures in Nucleic Acid Research*, vol. 2, p. 332. Ed. by G.L. Contoni and D.R. Davies. Harper and Row, N.Y.

Travis, J., D.J. Newman, J. LeGall, and H.D. Peck, Jr. 1971. The amino acid sequence of ferredoxin from the sulfate reducing bacterium *Desulfovibrio gigas*. Biochem. Biophys. Res. Comm. *45*: 452-458.

Trudinger, P.A. 1969. *In Advances in Microbial Physiology*, vol. 3, pp. 111-152. Ed. by A.H. Rose and J.F. Wilkinson. Academic Press, London.

Tuttle, J.H., P.R. Dugan, C.B. Macmillan, and C.I. Randles. 1969. Microbial dissimilatory sulfur cycle in acid mine water. J. Bacteriol. 97 *2*: 594-602.

Updegraff, D.M. and G.B. Wren. 1953. The release of oil from petroleum-bearing materials by sulfate reducing bacteria. Appl. Microbiol. *2*: 309-322.

Uspenskii, V.A., A.I. Gorskaya, and I.P. Karpova. 1947. Genesis of algarites and processes of anaerobic oxidation of petroleum. Izv. Akad. S.S.S.R., Ser. Geol. *4*: 89-106.

Vosjan, J.H. 1970. ATP generation by electron transport in *Desulfovibrio desulfuricans*. Antonie van Leeuwenhoek *36*: 584-586.

Winters, J.C., and J.A. Williams. 1969. Microbial alteration of crude oil in the reservoir. Symposium on Petroleum Transformations in Geologic Environments. Presented Before the Division on Petroleum Chemistry. IMC. American Chemical Society, New York Meeting, Sept. 7-12.

Yagi, T. and N. Tamiya. 1962. Enzymatic oxidation of carbon monoxide. Biochim. Biophys. Acta *65*: 508-509.

Yagi, T. 1958. Enzymatic oxidation of carbon monoxide. Biochim. Biophys. Acta *30*: 194-195.

ZoBell, C.E. 1947. Bacterial release of oil from oil-bearing materials, Part I. World Oil, Aug. 25: 36-47.

ZoBell, C.E. 1944. Annual report of progress. Fundamental research on occurance and recovery of petroleum. Am. Petrol. Inst., New York, N.Y.: 103-113.
(as described by Davis, 1967).

APPENDIX

Uptake and Metabolism of ^{14}C-1-Labelled Lactate by Isolate #1

This experiment was designed to study the metabolism of lactate, the major carbon substrate. Chemostat studies indicated that lactate, especially in the nutritional experiments, tended to be limiting. The results of the labelled yeast protein hydrolysate experiment described earlier indicate lactate is the primary electron donor (energy source) and provides the bulk of the cellular carbon for all synthesis.

The experiment described here was designed primarily to determine how much of the C-1 carbon of lactate was utilized (in the form of pyruvate) for purposes of cell synthesis, as well as to gain further evidence as to amounts of pyruvate oxidation to acetate and labelled CO_2 which might potentially occur.

The design of this experiment was as described in Materials and Methods, part VA. Media #'s 1-7 were tested. Medium #7 forced Isolate #1 to make use of lactate as the sole energy and carbon source. Each flask received 1.08×10^6 dpm of C-1 labelled lactate and 5 ml of 48 hour inoculum. Incubation was for 72 hours at 30°. The long incubation period was purposely used in order to ensure that the maximum amount of lactate oxidation to acetate and CO_2 would be allowed.

Table XXVII summarizes the amounts of labelled CO_2 which were produced during the growth of Isolate #1 on the seven media, the amounts of label incorporated, and the distribution of incorporated label. Table XXVIII summarizes the label incorporation and the production of labelled CO_2 as a percentage of the total labelled lactate added to each flask.

TABLE XXVII. INCORPORATION OF ^{14}C-1-LABELLED LACTATE INTO ISOLATE #1 CELLULAR COMPONENTS AND CO-PRODUCTION LEVELS OF $^{14}CO_2$ (1.08 x 10^6 dpm/culture)

Parameter Measured	Medium #						
	1	2	3	4	5	6	7
$^{14}CO_2$ found in KOH center well (dpm)	582,602	529,315	446,575	546,712	545,068	537,808	628,082
Label in Total RNA (dpm)	5,184	2,727	1,944	3,699	3,294	2,457	2,808
Total RNA/culture (µg)	306	450	270	324	468	504	234
RNA specific activity (dpm/µg)	16.9	7.2	11.0	-	7.0	5.4	12.0
Label in Total Protein DNA fraction (dpm)	21,760	23,420	15,240	11,960	16,840	17,780	26,020
Total protein/culture (µg)	13,000	18,200	14,800	14,000	20,400	17,800	8,000
Protein specific activity (dpm/µg)	1.6	1.2	1.02	0.85	.85	.99	3.25
0.3 N HC CO_4 wash (soluble pool) (dpm)	3,842	2,373	904	1,243	1,695	1,130	3,051
Culture Supernatant	278,100	396,300	395,700	256,800	367,200	426,000	361,428
Total label accounted	891,488	954,135	860,543	820,414	934,097	985,175	1,018,338
Percentage of Label Accounted	82.5	88.3	79.6	75.9	86.4	91.2	94.2

TABLE XXVIII. PERCENT OF THE ^{14}C-1 LABELLED LACTATE INCORPORATED INTO CELLULAR COMPONENTS OR FOUND IN METABOLIC CO_2

Medium #	% as CO_2	% as RNA	% as Protein-DNA	Total
1	53.9	0.48	2.0	56.38
2	49.0	0.25	2.1	51.35
3	41.3	0.18	1.4	42.88
4	50.6	0.34	1.1	52.04
5	50.4	0.30	1.6	52.30
6	49.7	0.22	1.6	51.52
7	58.2	0.26	2.4	60.86

The largest portion of the metabolized label appeared as $^{14}CO_2$. Depending on the growth medium used, 41-58% of the label could be detected as CO_2. This would indicate that at least 41-58% of the total lactate was completely oxidized to CO_2 and acetate. Unfortunately the media did not exhibit their growth differences as well as was demonstrated in the chemostat. This, no doubt, is due to the fact that the cultures were allowed to grow for a full 72 hours. By allowing this to happen, the cultures probably grew to rather similar levels at which point H_2S toxicity began occurring. Thus the H_2S levels in the sealed flasks exerted a greater control on growth in all likelihood than did the medium involved. This is a severe limitation of the experimental design, but could not be helped under the circumstances. Even more unfortunate was the fact that $^{14}CO_2$ production did not demonstrate any dilution effect due to the presence of various levels of unlabelled lactate in the 7 media tested.

It is not surprising that the specific activity of the protein was very low since only a few amino acids are derived from pyruvate (the only oxidation product of lactate still possessing the labelled C-1 carbon). These amino acids would comprise alanine, valine, leucine, and isoleucine. Small amounts of label were detected in the acid-soluble pool (0.3 N $HCl)_4$ wash). However, as in the case of the labelled yeast protein hydrolysate experiment, the amount of labelled pool was insignificant. This must mean, specifically in the lactate case, that by the time lactate carbon appears in the pool, it is mostly in the form of acetate and the carboxyl group has already been liberated as CO_2. Thus the organism does not appear to transport and maintain significant amounts of lactate or pyruvate within the cell during growth.

There were differences in the percentages of label accounted for in the 6 tests. Unless this can be explained by variance in the degree of $^{14}CO_2$ trapping among the flasks or other unknown quenching factors which were not considered, there is no explanation which would account for the lack of uniformity in the total accounting of label. The latter suggested possibility is unlikely since quench factors for the medium and for the KOH used for CO_2 trapping were calculated and used in all the labelled experiments.

The primary fact which was reinforced by this experiment was that lactate primarily serves as an electron donor; under most circumstances the lactate which is taken up is oxidized completely to acetate and CO_2. Further, although lactate can serve as the sole source of carbon for synthesis, only a small fraction is derived from pyruvate, while probably 90-95% of the remainder is derived from acetate (acetyl CoA).

Studies should be carried out, using C-2 and C-3 labelled lactate, to determine the amounts of ^{14}C incorporation into cellular materials.